BIOMARKERS OF GENOTOXIC AND REPROTOXIC EFFECTS AFTER CHEMICAL EXPOSURE

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Biomarkers of Genotoxic and Reprotoxic Effects after Chemical Exposure

The genotoxic effects due to the respiratory disease of Tuberculosis (TB) patients compared to healthy controls in diploid lymphocyte and haploid sperm cells, after treated with two heterocyclic amines and quercetin in bulk and nano forms.

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Abstract

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The genotoxic effects due to the respiratory disease of Tuberculosis (TB) patients compared to healthy controls in diploid lymphocyte and haploid sperm cells, after treated with two heterocyclic amines and quercetin in bulk and nano forms.

Keywords: Tuberculosis, lymphocyte, quercetin, nanoform, Comet assay, DNA damage, in vitro.

In the tuberculosis patients, *Mycobacterium tuberculosis* can stimulate production of hydrogen peroxide in the host as a result of immune response. The H_2O_2 accumulate in pulmonary cells, causing oxidative stress that could lead to the cancer. We select TB patients for this study which investigates the effects of quercetin as there is an increased incidence of latent TB among the migrant population in the past few years and TB can increase the risk of cancer.

Sperm and lymphocytes were treated with DNA damage inducers and quercetin (10μM, 25μM and 100μM), the responses evaluated using the Comet and micronucleus techniques. The gene expressions of COX1, COX2, P53 and Bcl-2 and catalase protein expression were investigated using the qPCR and Western blot techniques.

The results showed that a substantial reduction of DNA damage in lymphocytes from TB patients and sperm from healthy donors from * P ≤ 0.0283 to *** P≤0.001in the Comet assay. In the MNi assay, the effect of quercetin in lymphocytes was more significant in reduce DNA damage, whereas the DNA damage induced by a food mutagen was significant, from *p 0.0405 to ***p 0.001. The qPCR showed significance down-regulation of COX1 and Bcl-2 gene expression, rated between *p

0.045 and **p 0.0074. However, the catalase protein was up-regulated by the nano form of quercetin when using lymphocytes from TB patients and showed significant changes at *p 0.0236.

In conclusion, the nano form was found to be more efficient at the reduction of DNA damage in the Comet and micronucleus assays. Also, it down-regulated COX1 and Bcl-2 and up-regulated the catalase proteins indicating a possible role for quercetin, in genoprotection to TB through its enzyme modulating effect.

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Firstly, many thanks for my sponsor the Libyan Embassy for financial support and being offered a chance for this scholarship to complete my PhD. Secondly, I should like to offer my sincere gratitude and thanks to my supervisor Prof Diana Anderson, for her help, continuous support, encouragement, and advice. I have appreciated her efforts that have support me every day. I am deeply indebted to her helping in the most critical steps of my research. Also, special thanks to Dr. Mojgan Najafzadeh and Professor Badie Jacobs for their help in providing the blood samples.

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Abbreviations

Alkali-labile site (ALS)

Binucleated (BiNC)

Bleomycin (BLM)

Bovine Serum Albumin (BSA)

Cyclooxygenase enzyme 1(COX-1)

Cyclooxygenase enzyme 2(COX-2)

Cyclooxygenases (COX)

Cytochalasin-B (Cyto B)

Cytokinesis Block Micronucleus assay (CBMN)

Deoxyribonucleic acid (DNA)

Dimethyle sulfoxide (DMSO)

Dithiothreitol (DTT)

Double strand break of DNA (DSB)

Enhanced chemiluminescence (ECL)

Ethylene diamine tetrachloro acetic acid (EDTA)

Foetal bovine serum (FBS)

Hydrogen peroxide (H_2O_2)

Hydroxy propyl Methyl cyeluse (HPMC)

Low melting point agarose (LMP)

Micro molar (µM)

Micronucleus (MNi)

Mitomycin C (CMM)

Nano gram (Ng)

Nanoparticles (NPs)

Negative control (NC)

Nitrocellulose and Polyvinylidene fluoride (PVDF)

Normal melting point agarose (NMP)

Nuclear buds (NBUDs)

Nucleoplasmic bridges (NPB)

Olive tail moment (OTM)

Phytohaemagglutinin (PHA)

Poly Vinyl pyridine (PVP)

Polymerase chain reaction (PCR)

Positive control (PC)

Reactive oxygen species (ROS)

Single strand break (SSB)

Single strand break of DNA (SSB)

Sodium chloride (NaCl)

Sodium chloride (NaCl)

Sodium Dihydrogen phosphate (NaH_2PO4)

Sodium dodecyl Sulphate (SLS)

Sodium hydroxide (NaOH)

Sodium hydroxide (NaOH)

The nuclear division index (NDI)

Thioredoxin (Trx)

Tuberculosis (TB)

Western blots (WB)

1 Chapter (1) Introduction

Introduction

Biomarkers can be defined as characteristics that are calculated and estimated accurately as a mark of a normal biological process (Strimbu and Tavel, 2010). Biological characteristics deal with a broad subcategory of medical symptoms; that is, impartial marks of a medical state observed from outside the patient's body, which can be calculated correctly and reproducibly in genetics (Ochekpe et al., 2009). Genotoxicity represents the characteristics of a chemical or physical material that damages the DNA within a cell, which could cause mutations that can potentially progress to cancer; however, genotoxicity is often confused with cell mutagenicity. Somatic or germ cells of the organism can be affected by permanent heritable changes, which can be passed on to new generations (Nagarathna et al., 2013). Cellular metabolism process can stop chemically-induced mutations by either apoptosis or DNA repair; however, this system may not always function normally, leading to mutagenesis (Brendler-Schwaab, 2005). Interestingly, some current drug improvement efforts are focusing on DNA repair as a way to monitor malignancy. More specifically, nanotechnology has generated a new dynamic in the pharmaceutical and medical sciences due to its ability to improve many intrinsic properties of materials at the nano-scale (Chen et al., 2009). This type of technology extends the field of pharmaceutical preparations presently used in cancer treatment as their properties might be improved by a decreased particle size. Promising results have already been obtained in our laboratory using aspirin and ibuprofen nanoparticles (NPs) when compared to bulk substance in cancer prevention and treatment, which is the primary focus of the present research on these NPs for the

Treatment of various diseases.

1.1 Genotoxicity

Eastmond et al. (2009) explained that genotoxicity is related to damage or changes in genetic material and structure that may be connected with mutagenicity in some cases. This includes gene mutations, such as insertions, deletions, and point mutations, as well as DNA strand breaks. These breaks lead to chromosomal structural changes, which is also referred to as a clastogenic effect, and the induction of a DNA enzyme that is responsible for the formation of DNA adducts by chemical or physical substances with genotoxic properties, they can cause genetic and substitutions that lead to additional mutations and increase the risk of inflammation, congenital defects, and cancer. Therefore, genetic toxicology has become a crucial component of laboratory research related to drugs and other compounds to examine the impact of chemicals, and physical agents.

1.2 Lymphocytes

Lymphocytes are a type of white blood cell that originates from stem cells in the bone marrow. Lymphocytes are cells of the immune system, living in lymph nodes, also can be present in the blood stream and body organs. There are three types of lymphocytes B cells, T cells and natural killer cells (Verywell Health, 2018). Lymphocytes can be used to study the effects of genotoxic compounds because lymphocytes may reflect cancer cells and other disease states (Anderson, 2014; Najafzadeh et al., 2012). Henderson et al. (1998) explained that lymphocytes should be examined for viability 30 minutes after a treatment to avoid cytotoxic effects of chemical compounds. Viability should be above 75% using the trypan blue stain test, which is very useful for unknown chemicals to avoid false positive results in the

Comet assay. In 2000, the World Health Organization suggested that viability changes, and 50% viable cells measured via trypan blue are considered viable (World Health Organization, 2000). However, 75% was preferred and confirmed as a cut-off point to avoid false positives (Henderson et al., 1998). The fact that even untreated lymphocytes can display 0–10% DNA in the tail (Collins, 2004), and sperm can exhibit up to 20% DNA in the tail (McKelvey-Martin et al., 1997) should also be taken into consideration during interpretation of results.

1.3 Respiratory disease

The present study focuses on tuberculosis, which is one from respiratory diseases that including lung cancer, chronic pulmonary obstructive disease (COPD) and asthma, which are common diseases that threaten human health. Our laboratory studies biomarkers of genotoxic and reprotoxic effects after chemical exposure. We use human lymphocytes, whole blood, and sperm samples from healthy and disease-state individuals. In this study, we are examining cells from TB patients. Since asthma, chronic obstructive pulmonary disease (COPD) and lung cancer have already been examined to some extent, we decided to investigate tuberculosis patients, with DNA damage induced in lymphocytes by genotoxic substances such as, hydrogen peroxide or food mutagens and treated with nano and bulk forms of quercetin. Since many disease states involve oxygen radical damage, we used quercetin, a flavonoid, which is known to reduce free radicals. Tuberculosis is an infectious disease, but humans can control it by avoiding direct contact with TB patients and changing their lifestyle, engaging in regular exercise, avoiding smoking, and maintaining a healthy diet. Fruits and vegetables have been found to play a protective role by inhibiting inflammatory diseases and cancer transformation (Van

Duyn and Pivonka, 2000). Moreover, many people with chronic respiratory disease and tuberculosis are at risk of developing lung cancer (Yu et al., 2011).

Tuberculosis (TB)

TB is an infectious disease caused by bacilli bacteria named *Mycobacterium tuberculosis* and is known to have existed for more than 17,000 years. It spreads during contact with infected patients, especially after sneezing or coughing. Moreover, TB has been categorised as one of the ten most infectious diseases and is a major cause of death globally (Sandhu, 2011). About 9.6 million people were diagnosed with TB in 2014: 3.2 million women, 5.4 million men, and 1.0 million children. Additionally, HIV-positive patients comprised 12% of the total TB patient population in 2014 (WHO, 2014). Approximately 1.4 million people die due to TB infection annually (Petti, 2016; Zumla et al., 2015). The most important organ of the human body affected by TB is the lungs, but the bacteria can also infiltrate other areas of the body, such as the nervous system and bones. TB is more deadly in elderly and HIV-positive patients. Generally, TB patients have symptoms such as chest pain, weight loss, cough, and difficulty breathing, and these patients more likely to transmit the disease (World Health Organization, 2014). Although TB is present in nearly every country, Cayla and Orcau have reported that London is the TB capital of Europe (Caylà and Orcau, 2011).

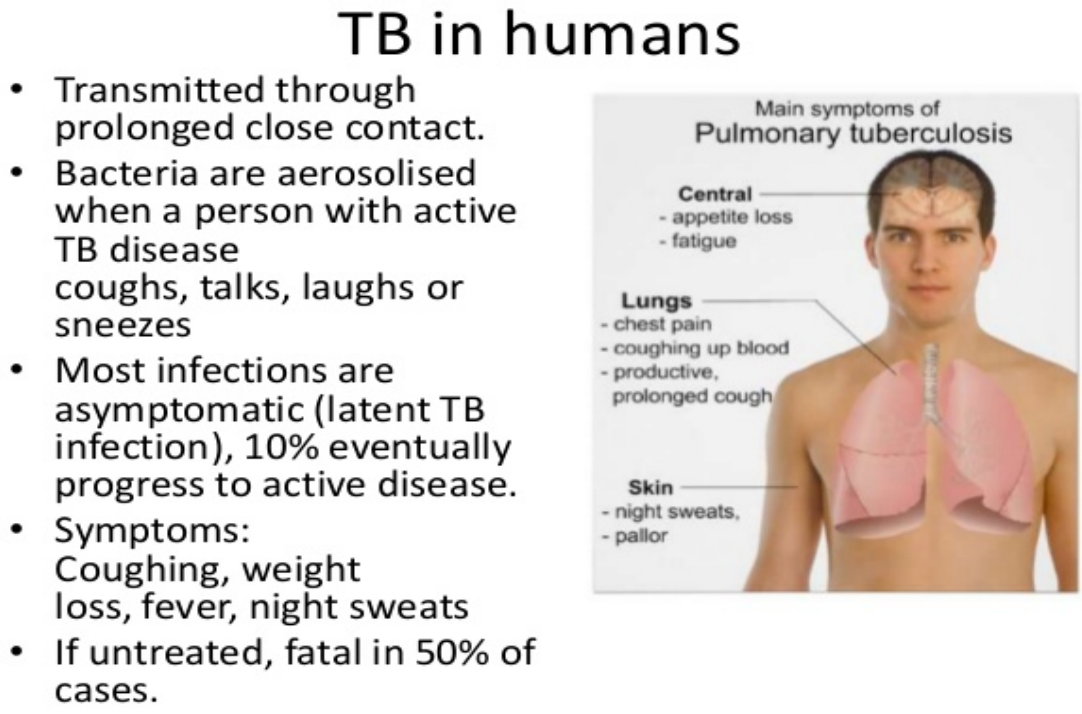


Figure 1: Symptoms and sings of TB in human body (Image.slidesharecdn.com, 2019).

1.3.1.1 Tuberculosis and DNA damage

Lung cancer (LC), Chronic Obstructive Pulmonary Diseases (COPD) and Tuberculosis (TB) have been studied to evaluate DNA damage and the frequency of cellular abnormalities in LC, COPD, and TB, patients by comparing them to healthy Individuals. Results showed that The COPD group had a high percentage of apoptotic cells compared to TB and LC group, whereas the TB group presented a higher incidence of DNA damage, apoptotic and necrotic cells. Patients with Lung cancer had a lower frequency of chromosomal abnormalities than COPD and TB patients (da Silva et al., 2015). Increased oxidative stress in the lung tissue of elderly people leads to an escalation in the activity of free radicals, which damages the DNA of pulmonary and blood cells (Valavanidis et al., 2013). The interactive influence of

breathable molecules leads to oxidative stress. These molecules have a high carcinogenic potential and can lead to the increased production of pulmonary inflammatory mediators, which are thought to cause oxidative damage to all cellular components (e.g., DNA, proteins, and membrane lipids) (Valavanidis et al., 2013). The chemical and physical properties of molecules, including their size and shape, as well as stable free radicals and transitional metal content play a major role in oxidative stress damage. Oxidative stress starts the synthesis of mediators of pulmonary inflammation in lung tissues and stimulates carcinogenic mechanisms (Valavanidis et al., 2013). It has been stated that the progression to cancer from pulmonary disease is connected with a different biological pathway of DNA oxidative damage, macrophage activation, and gene expression modifications that stimulate transcription factors and play a significant role in carcinogenesis (Valavanidis et al., 2013). Conversely, some types of bacteria, like *Mycobacterium tuberculosis*, producing free radicals (H_2O_2) in the host as a result of numerous defence mechanisms (Ng et al., 2004). DNA repair has been examined as a significant mechanism for the survival of *Mycobacterium tuberculosis* in the host (Kurthkoti and Varshney, 2012). *Mycobacterium tuberculosis*-inducible DNA repair genes which are arranged via LexA (Protein) homologs, which depend on RecA (Protein). Studies have examined RecA's involvement in two major pathways, which are the promoters of gene expression following the DNA damage present in *Mycobacterium tuberculosis* (Davis et al., 2002).

1.3.1.2 Mechanism of DNA damage in Tuberculosis

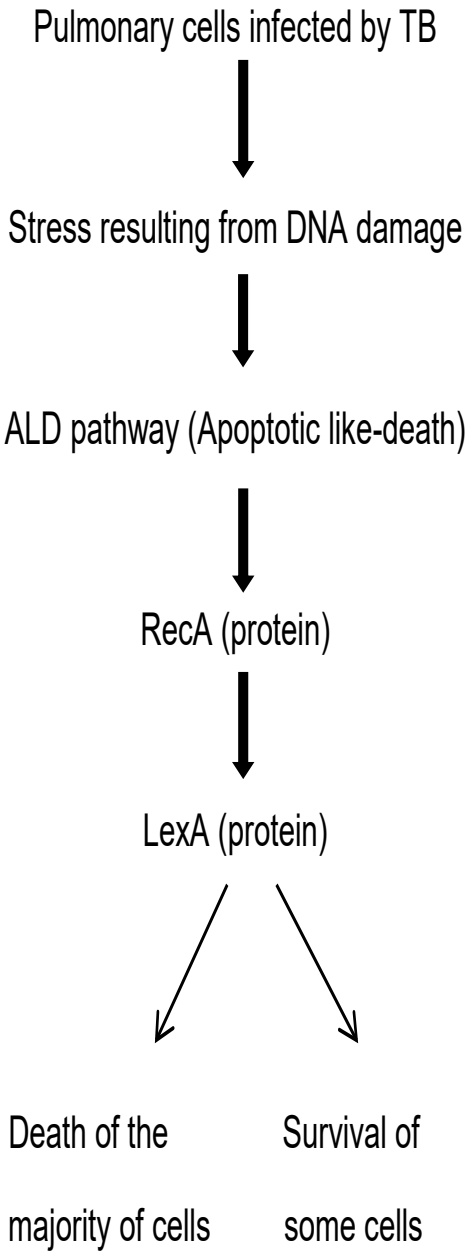


Figure 2: Mechanism of DNA damage in tuberculosis adapted on 22/12/2018 from (Davis et al., 2002).

1.4 Genes related to study

1.4.1 Cyclooxygenase enzyme (COX)

Cyclooxygenase (COX) is the principal enzyme in the biosynthetic pathway of prostaglandins. The enzyme changes free arachidonic acid originating from membrane phospholipids using phospholipase A2 to produce prostaglandin H2 and, finally, prostaglandins and thromboxane. There are two isoforms of COX, which are COX1 and COX2 (Gao et al., 2014). COX1 can be found in any site of inflammation; it has the ability to maintain tissue homeostasis and regulates angiogenesis. Both COXs are responsible for prostaglandin synthesis and use a similar pathway. Nagao et al. (2013) reported that COX1 and COX2 are encoded by different genes found on separate chromosomes, and their expression pattern is completely different. COX1 is substantially expressed in general tissues and is responsible for the synthesis of prostaglandins involved in various housekeeping functions, such as in the gastrointestinal tract and the regulation of renal and platelet functions. COX2 can be immediately activated by an inflammatory stimulus and produces a large number of pro-inflammatory cytokines and mitogens that increase the amount of prostaglandins and affect cell proliferation, angiogenesis, and apoptosis, which finally lead to malignant transformations (Kuroda and Yamashita, 2002). Furthermore, a study has illustrated that genetic polymorphisms of both COXs are associated with developing cancer and affecting drug efficacy (Nagao et al., 2013). Different studies have examined links between changes in COX1 and COX2 gene expression and the effect of nonsteroidal anti-inflammatory drug (NSAID) intake on cancer risk; they have reported conflicting results but confirmed that the COXs' expression depends on the tumour's aggressiveness and size, as well as lymph node enlargement

(Nagao et al., 2013; Gao et al., 2014). Decreased COX-2 expression could inhibit the proliferation of cancer cell lines in vitro (Davis et al., 2012). Yu et al. (2010) noted that genetic polymorphisms of COX-2 change its expression and affect the carrier's susceptibility to different carcinomas.

1.4.2 Tumour protein p53 (Tp53)

TP53 is classified as a tumour suppressor and considered 'the protector of the genome' because it is responsible for many cellular pathways, such as cell cycle arrest, apoptosis, and DNA repair (Kim et al., 2015). The importance of p53 in cancer inhibition is evident; however, the results have been controversial as it is inactive in about 50% of the total malignancy (Bieging et al., 2014). Our understanding of p53's role in vivo is clear in the elderly. In a modified mouse, a shortened form of p53 that enhances wild-type p53 activity improved resistance to spontaneous malignant transformation. Wilson et al. (2010) demonstrated that p53 expression is reduced in neural progenitor cells and mesenchymal stem cells and related to age and p53's effectiveness is reduced when age is increased at the organismal level (Feng et al., 2007). The results generally indicate that the loss or reduction of p53 expression, particularly in the elderly, leads to the development of cancer (Kim et al., 2015). Furthermore, one of p53's most important functions is its ability to trigger apoptosis, and quercetin can induce apoptosis in cancer cells through the upregulation of Bax and Bak and the downregulation of Bcl-2 (Gibellini et al., 2010).

1.4.3 B-cell lymphoma 2 (Bcl-2)

The Bcl-2 protein is from the Bcl-2 family that regulates programmed cell death (apoptosis) and can be referred to as B-cell leukaemia/lymphoma 2 protein (Papadopoulos, 2006). This protein helps control whether a cell lives or dies by

blocking a pattern of apoptosis. The gene for Bcl-2 is located on chromosome 18, and the translocation of the Bcl-2 gene to various chromosomes is determined in different types of leukaemia and lymphomas (Youle and Strasser, 2008). Bcl-2 can monitor apoptosis by controlling mitochondrial external membrane permeability, which is the key point in the apoptosis pathway (Papadopoulos, 2006). Furthermore, quercetin is considered a generator of apoptosis in cancer and inflammatory cells by triggering apoptosis through the mitochondria pathway; the quercetin can release the cytochrome c from mitochondria to cytosol that activates two types of caspases, caspase -3 and caspase-7 which guide cells to apoptosis. Quercetin adjusts anti-apoptotic and pro-apoptotic proteins. Specifically, quercetin promotes the upregulation of Bak and Bax and downregulation of Bcl-xL and Bcl-2. Once the bcl-2 is downregulated apoptosis may have increased therefore detecting the multimerization of Bax to the membrane of mitochondria. These steps are then joined by the division of procaspase 9 and poly (ADP-ribose) polymerase (PARP) (Gibellini et al., 2010).

1.4.4 Catalase

Catalase was unknown until 1818 when Louis Jacques, who identified hydrogen peroxide, suspected its breakdown is due to a foreign material. Oscar Loew (1900) was the first scientist to use the name catalase. In 1937, catalase from cow liver was isolated is a crystallised form by James B. Sumner and Alexander Dounce, and the molecular weight of catalase was detected in 1938 (Sumner and Gralen, 1938). In 1969, the amino acid structure of bovine catalase was determined (Schroeder et al., 1969). Lastly in 1981, the protein's three-dimensional structure was discovered.

The human catalase gene is positioned on the small arm of chromosome 11 and has all the properties of a housekeeping gene. Also, catalase is displayed in all human / animal body tissues, especially in the liver, kidney, and red blood cells. Cancer cells usually produce high levels of ROS, which could be due to defects in mitochondria functions and reduced antioxidant enzyme production, such as catalase. Low levels of catalase have been observed in different genetic diseases, for example, non-small-cell lung cancer, allele loss, chromosome 11p deletion, mental retardation, and Wilms tumours (Glorieux et al., 2015). Habas et al. (2018) studied DNA damage using Comet and micronucleus assays in peripheral blood lymphocytes collected from chronic pulmonary obstructive disease (COPD) patients and compared the results with lymphocytes collected from healthy individuals. The lymphocytes were treated with nanoparticle (NP) and bulk forms of the flavonoid quercetin. mRNA and protein expression levels of catalase have also been investigated using qPCR and Western blot techniques. Furthermore, changes in oxidant-antioxidant inequality of the catalase gene have been examined in connection with the accumulation of oxidative stress in peripheral lymphocytes collected from patients with COPD. The results showed that lymphocytes where DNA damage was induced by 140 μM IQ and treated with 10, 25 μM quercetin bulk and nano forms for 30 mins. The significant decreases in DNA damage (***$p \leq 0.001$) was shown using the Comet and micronucleus assays. Also, Habas and colleagues studied the regulation of the catalase enzyme activity by IQ at the protein or gene expression levels using the Western blot and qPCR techniques; they found that the quercetin nano form was more effective in terms of reducing catalase protein and relative catalase mRNA expression (Habas et al., 2018).

1.5 Spermatogenesis

Hess (2005) showed that sperm production is the process that produces male haploid germ cells from stem cells called diploid spermatogonia, which is shown in Figure 3. The procedures have been simplified morphologically through recognising associated cellular steps and phases of spermatogenesis that are vital for continuous sperm production; this development can be dependent on several factors, including joining paracrine and autocrine (from Sertoli and germ cells) and endocrine function (e.g., androgens, retinoic acids) (Hess and Franca, 2005). Spermatogenesis can be described as the production of haploid spermatozoa from diploid spermatogonial (primordial) male germ cells via both mitotic and meiotic cell division. The main cells in these stages are called spermatogonia, which produce spermatocytes by mitosis. These processes are controlled by hormones and cell to cell reaction (germ –and Sertoli cells) to produce final sperm. Three stages of spermatogenesis occur in the mammalian cells in the testes (seminiferous tubules), which require about 12 weeks to complete in humans. Spermatogenesis begins with a proliferation stage during which primordial cells undergo multiple mitotic divisions that produce stem cells of spermatogenesis (gonocytes). Once the differentiation of gonocytes is complete, spermatogonia are created. Next, spermatogonia proliferation occurs through several mitotic cell divisions. Some spermatogonia cells can differentiate to produce primary spermatocytes, while other cells continue proliferating. After primary spermatocytes divide in the meiosis stage, secondary spermatocytes are shaped and divide into haploid spermatids, which develop into mature spermatozoa (Marchetti and Wyrobek, 2005). The spermatogenesis process takes approximately 74 days. However, studies of healthy men have indicated that the total time required to produce sperm is 42–76 days. The expected daily sperm

production per human male fluctuates between 150–275 million spermatozoa (Neto et al., 2016). During this time, some factors, such as nutrition and hormones, can cause DNA damage in sperm (Appasamy et al., 2007). Other factors can influence spermatogenesis, for example, alcohol consumption, some drugs used to treat chronic diseases, an inadequate diet, and oxidative stress (McKay et al., 2015). The following diagram demonstrates the spermatogenesis process (Mudalir, 2015).

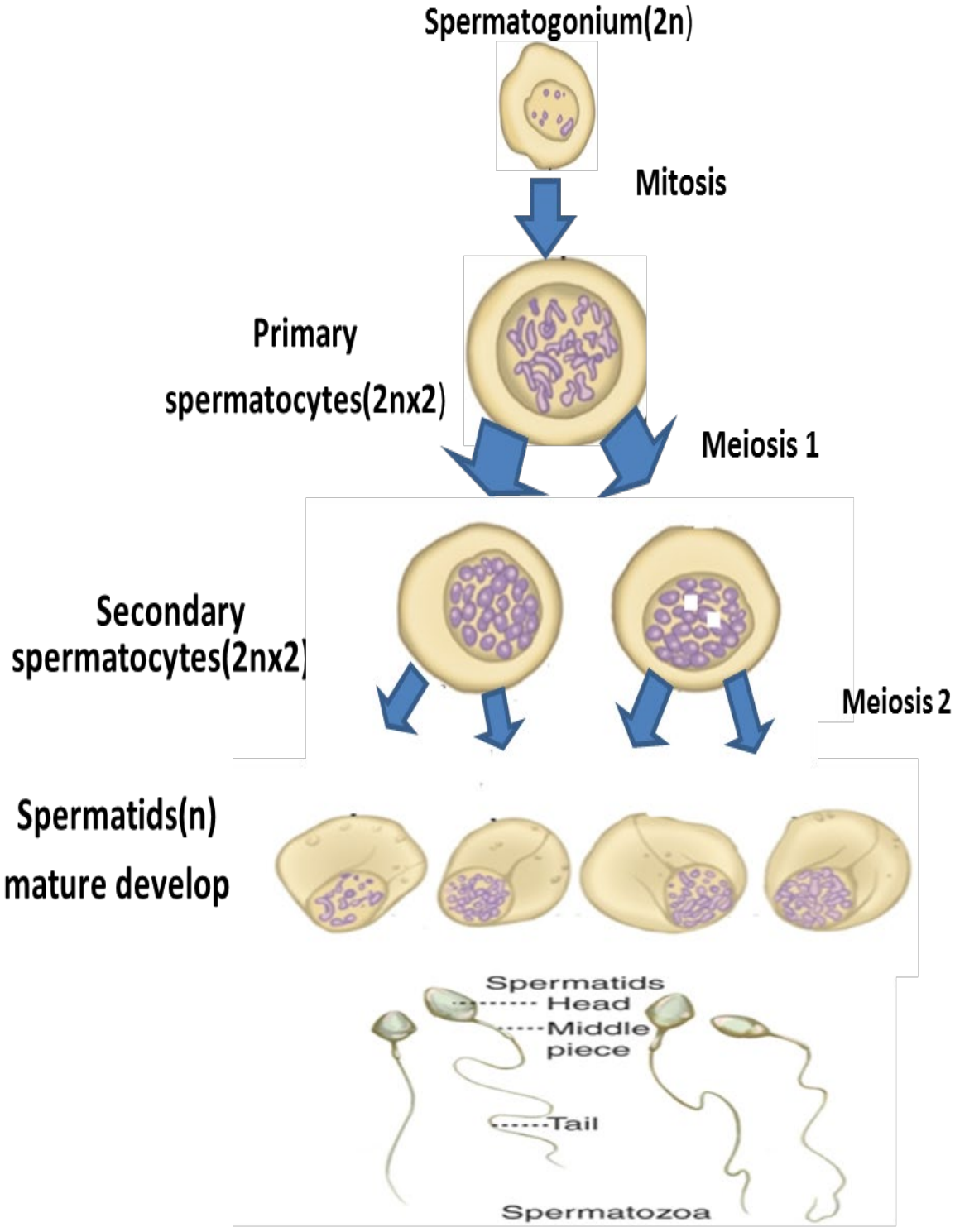


Figure 3: Spermatogenesis processes adapted on 25/12/2018 from (Mudalir.,2015).

1.6 Chemicals used in this study

1.6.1 Quercetin

Reactive oxygen species (ROS) can be formed in living cells due to low antioxidant capacity or an escalation of oxidative stress. Excessive oxidative stress has been shown to be responsible for DNA damage in cellular macromolecules, including proteins and lipids (Nordberg and Arner, 2001; Valko et al., 2007). The creation of ROS can produce single and or double strand breaks in the DNA and causes a change in the chemical structure by substituting pyrimidine bases, 2′-deoxyribose, and purine (Hazra et al., 2007). Bjelland and Seeberg (2003) reported that DNA damage from oxidative stress is a vital promoter of cancer transformation, especially in the elderly. Base excision repair (BER) can modify this type of DNA damage, and specific types of oxidative lesions can be adjusted by mismatched repair (MMR) and nucleotide excision repair (NER) (Dusinska et al., 2006; Svilar et al., 2011). Fruits and vegetables are known to have antioxidant properties, which have a significant impact on inhibiting DNA damage and malignant transformation. One of the most common compounds in fruits and vegetables are flavonoids, which have been widely studied; there is a strong indication that they work as antioxidant compounds or pro-antioxidants depending on their ability to react with food mutagens and their concentrations to reduce DNA damage (Duthie et al., 1996; Anderson et al., 1998). Flavonoids can be obtained in significant amounts from the daily diet and offer anti-inflammatory and anti-oxidant benefits (Serafini et al., 2010). Flavonoids like quercetin (Q) can be obtained from the rinds and bark of several types of fruits and plants, and the consumption of apple skins, onion bulbs, and the dry outer skin of onions is highly recommended (Hard et al., 2007). The evaluated average dietary consumption of quercetin is mainly as glycoside at 0.1–0.2 mg/Kg. It has been

shown that quercetin is carcinogenic in male rats at very high concentrations; it induces renal adenomas after feeding at 2000mg/Kg for 6 months to 2 years (Hard et al., 2007). In combination with hydrogen peroxide the quercetin decreases the DNA damage level (Gschwendt et al., 1983). Quercetin is known by several chemical names, including CAS 117-39-5, sophortin, meletin, xanthaurine, and quercetol; its molecular formula is $C_{15}H_{10}O_7$, and its molecular weight is 302.2357g/mol.

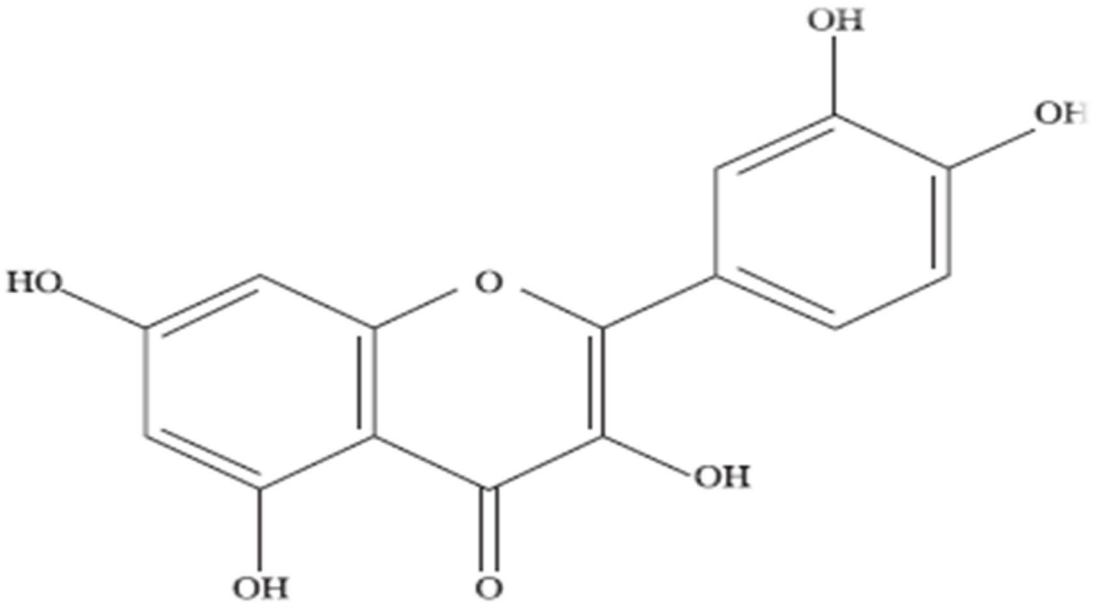


Figure 4: Quercetin chemical structure (McKay et al., 2015).

Sources of flavonoids vary between countries. In South Europe, red wine is the best source, while in China green tea is the most common source of flavonoid; however, in the United States, apple, onion, and fermented food, is the main source of flavonoid compounds. In 2007 it was reported that the human body needs 5-40 mg of quercetin/day (Wach et al., 2007, Russo et al., 2012). Another type of flavonoid such as rutin which has a high ability to reduce blood pressure due to decreases in

the permeability of blood vessels and scavenging free radicals, these flavonoids are widely used in different countries (Li et al., 2001).

1.6.1.1 Quercetin mechanism

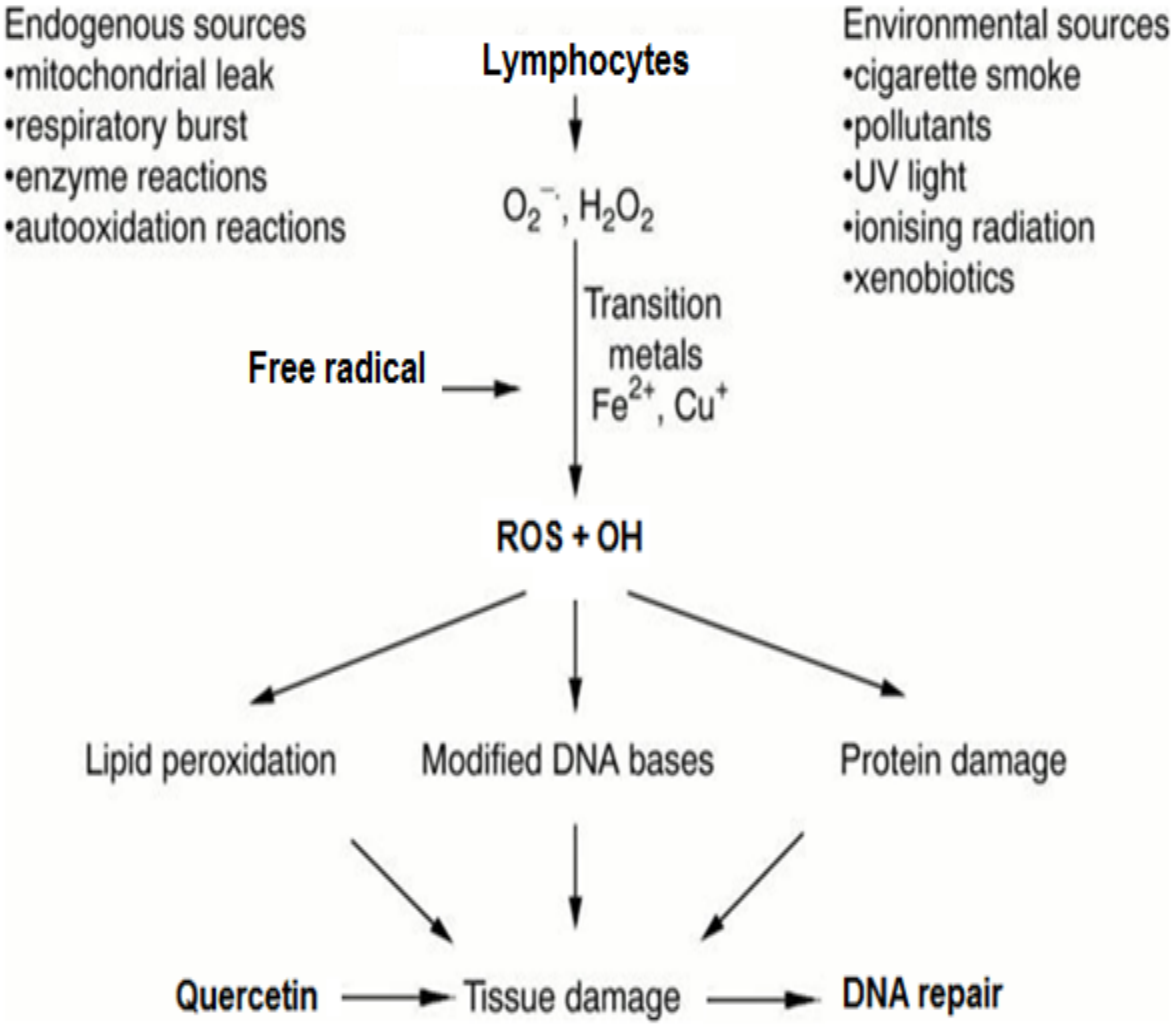


Figure 5: Mechanism of quercetin in reducing DNA damage (Shalini Kapoor et al., 2015).

Localised levels of oxidative stress are due to dissimilarity of the two anions (superoxide) from oxygen and H_2O_2, while quinones and semiquinones can also damage DNA directly (Bolton, 2002). Consequently, the production of free radicals at

high levels may cause DNA damage in a pro-oxidative state, resulting in chromosomal instability (Gibellini et al., 2010). However, quercetin has a strong effect in scavenging free radicals by inhibiting lipid peroxidation (Bentz, 2009). Following that the oxidation of free radicals which are catalyzed by molecular oxygen to produce lipid peroxy radicals, the continuum in the reaction induced by additional hydrogen from unsaturated fatty acid to produce more free radicals where the metal ions catalyzed the processes (Bentz, 2009). The toxic features of lipid peroxidation can cause cardiovascular disease and malignant transformation, but antioxidants, such as quercetin and myricetin can block the reaction by scavenging free radicals (Bentz, 2009). Studies have confirmed that quercetin has the ability to stop low density lipoprotein (LDL) oxidation, which is responsible for cardiovascular diseases and arteriosclerosis (Hollman et al., 1999). It has been shown that oxidative stress causes the accumulation of amyloid beta-peptide in brain membrane lipids, which leads to Alzheimer's disease; thus, the regular intake of antioxidants can prevent amyloid synthesis (Ansari et al., 2009). In this case, quercetin can increase the amount of glutathione (GSH), which has a significant role in the neuron defines. Furthermore, GSH can prevent the formation of H_2O_2 by separating H_2O_2 into H_2 and O_2 and stop free radical production (Balazs and Leon, 1994). Quercetin can also regulate the development of inflammation because it stops free radical production and transcription factors that are responsible for generation of pro-inflammatory cytokines, which are formed in patients with chronic inflammatory diseases (Boots et al., 2008). Environmental factors can also increase the production free radicals, such as smoking, and it has been found that flavones, which are the principle structure of quercetin, can protect erythrocyte membrane that is damaged by smoking (Begum and Terao, 2002). As indicated previously, quercetin induces apoptosis via ROS

generation and through the subsequent activation of the ROS/ AMPKα1 and AMPK α1/COX2 signalling pathways (Lee et al., 2008). COX1 can be present in any site of inflammation (Kuroda and Yamashita, 2002). Quercetin activates apoptosis by balancing pro-apoptotic and anti-apoptotic proteins and upregulates Bax and Bak, but it downregulates Bcl-2. Hence, it was decided to study the gene expression of COX1, COX2, Bcl-2, and p53.

1.6.2 Hydrogen peroxide (H_2O_2)

Hydrogen peroxide is a chemical compound with the formula H_2O_2, which mean two hydrogens and two oxygen molecules are connected together This compound has an oxygen-oxygen single bond and can be used as an oxidiser, and in high concentrations, it leads to formation of reactive oxygen species (Chapurkin, 2016).

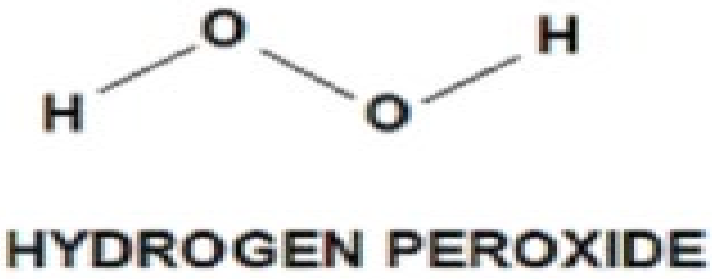


Figure 6: Chemical structure of hydrogen peroxide (Chapurkin, 2016).

Hydrogen peroxide is commonly used as a DNA damage inducing agent at 50μM on human lymphocytes and caused significant DNA damage detected by the comet assay (Anderson et al., 1994). The most important oxygen-containing free radicals in

several disease conditions are H_2O_2 and superoxide anion radical, but the level of free radicals and other reactive oxygen species in the human body can be controlled by antioxidant compounds like quercetin (Lushchak, 2015). Sierens et al. (2002) reported that H_2O_2 was used to induce sperm DNA damage after pre-treatment with antioxidants; they used a Comet assay to evaluate DNA damage and found significant protection against DNA damage at 100μM.

1.6.3 Bleomycin

Bleomycin (BLM) is a natural glycopeptide anti-cancer drug synthesised from Gram positive bacteria called *Streptomyces verticillu*. Bleomycin was discovered in 1976 by Umezawa in Japan. The drug has a unique composition and method of action amongst chemotherapy treatments (Sikic et al., 1985). It can be used to treat different types of cancer, such as Hodgkin's lymphoma, non-Hodgkin's lymphoma, ovarian cancer, cervical cancer, and testicular cancer. Chen et al. (2008) demonstrated that the drug has anticancer properties because it has the ability to alleviate DNA damage with participation factors such as Fe (II), O_2, and a one-electron reduction. Many hypotheses have been proposed to explain BLM's mechanism of action, and one study demonstrated that it has the ability to stop the combination of thymidine into DNA (Murray et al., 2018). Moreover, BLM can produce metal-bleomycin when it reacts with metal ions, which then combine with O_2 to produce free radicals (i.e., hydroxide and superoxide), which lead to single and double strand breaks (SSB, DSB). Another study suggested that BLM may affect certain locations on the DNA strand and persuade cleavage by reducing the hydrogen atom from the nitrogen base, which leads to strand breakage (Chen et al.,

2008; Huls and ten Bokkel Huinink, 2003). BLM can connect to DNA at specific locations and operates as a mini enzyme after creating a compound from O_2 and iron that forms free radicals that are responsible for DNA strand breakage (Sikic et al., 1985). Other studies have reported that chromosomal breaks increased significantly during the treatment period when BLM was used as an anti-cancer agent (Hu et al., 2013).

1.6.4 Food mutagens

There is substantial evidence that dietary factors contribute to cancer risk in humans; approximately 30–40% of cancers are thought to be influenced by dietary substances (Doll and Peto, 1981; Popkin, 2007). Epidemiological studies have provided most of the evidence regarding diet and cancer development (Wasson et al., 2008). In 1970, Sugimura and others worked on the heterocyclic amines (HCAs) and showed that pyrolysates of amino acids of foods contained high proteins (Knasmuller et al., 1999). Researchers published several studies to clarify how these compounds negatively impacted human health. Heterocyclic amines 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) and 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) are both carcinogens that cause DNA damage (Overvik and Gustafsson, 1990; Sugimura, 1997).

1.6.4.1 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)

PhIP is one of the most significant heterocyclic amines formed when meat is cooked. This compound (see Figure 7) is considered carcinogenic and could be involved in cancer development (Sugimura,1997).

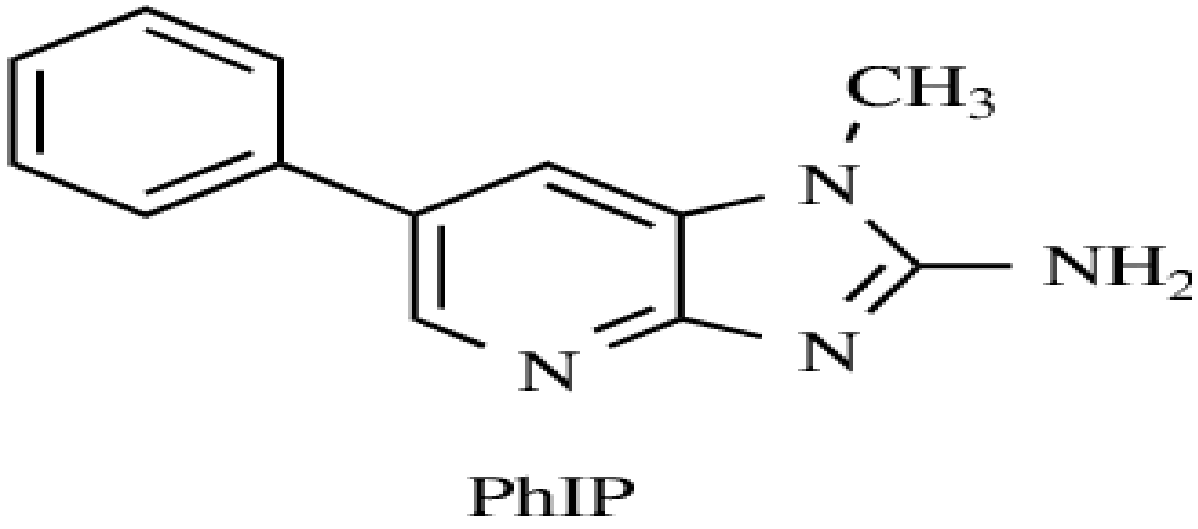
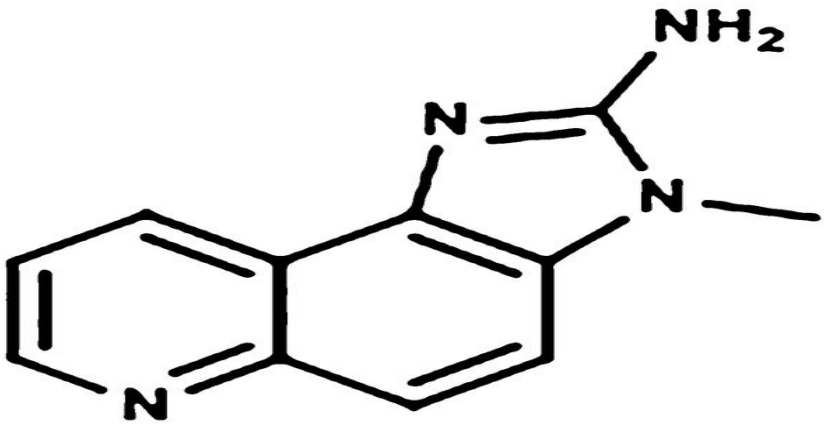


Figure 7: https://www.researchgate.net/figure/Fig-4-Structures-of-PhIP [accessed 8 Oct 2019]

The generation of PhIP is a result of a complex reaction in the presence of monosaccharides, which play a role in the Maillard reaction (Skog, 1993). Another PhIP source is smoking tobacco (Ushiyama et al., 1991). Peluso et al. (1991) demonstrated that DNA adducts related to PhIP could be isolated from smokers' urine. PhIP stimulates the development of lymphomas, colon cancer, and breast cancer in rats (Ito et al., 1997), although cells have a defense mechanism against PhIP with compounds that eliminates toxicity and repair DNA damage. The actions of phase II enzymes, such as UDP-glucuronosyltransferase (UGT), are specific to removing PhIP toxicity through the production of easily absorbed compounds, such as N-OH-PhIP-N2-glucuronide (Bromberg, 2000; Groner and Gouilleux, 1995). The effect of PhIP genotoxicity leads to mutations, including DNA damage and chromosome aberration, and increases sister chromatid exchange (Otsuka et al., 1996; Wu et al., 1997). Unsuccessful DNA repair processes could develop to cancer, especially once damage occurs in tumour suppressor genes and proto-oncogenes (Aboyade-Cole et al., 2008)

1.6.4.2 2-Amino-3methyylimidazo [4, 5-f] quinolone (IQ)

The heterocyclic amine IQ was discovered in 1980. IQ can be formed by cooking proteinaceous food (Felton and Knize, 1991). The daily intake of heterocyclic amines can reach 50 µg or 70 ng/g of meat (Knizeetal., 1995; Kruletal., 2000).



IQ

Figure 8: Chemical structure of IQ 2-Amino-3 methylimidazo [4, 5-f] quinoline (Han et al., 2008).

Endo et al (1994) studied the effect of PhIP and IQ food mutagen on fibroblasts isolated from the human body, The dominant mutation originated with IQ and PhIP was G:C to T:A trans version, then G:C to A:T conversion, also G:C to C:G trans version (Endo et al., 1994). Moreover, IQ caused mutations in the Ki-ras or p53 gene; this genetic alteration has been identified in colon carcinogenesis (Huang et al., 1996; Kakiuchi et al., 1995). IQ was observed in cigarette smoke at 0.26 ng per cigarette (Yamashita et al., 1986). It has been reported that IQ promotes colon tumours in rats and liver tumours in monkeys via base substitution. IQ was expected to cause base deletions, but the effects were not acute, site-specific, or unique (Kakiuchi et al., 1995). However, in combination with food mutagens, flavonoids

have produced antigenotoxic effects since DNA damage was reduced in lymphocytes and sperm when analysed using a comet assay (Anderson et al., 1998).

1.7 Nanotechnology

Nanotechnology is the manufacturing, characterisation, and use of materials at a measure of one-billionth of a metre (10^{-9}). Materials at these measurements have been used in several aspects of computer, medical, and engineering sciences. The effectiveness of NPs depends on their ability to remove the size-related barriers of a substance. When a substance is decreased to the nano size, it displays new characteristics, such as changes in their biological and physicochemical properties, compared to bulky substances (Ochekpe et al., 2009). Researchers have been able to understand these particles and assess advantages of their valuable characteristics at this scale (Chan, 2006). When materials change from their bulk form to a NP form, their solubility and surface area increase, as well as their immunogenicity and drug delivery properties, due to improved contact between the NP and the surrounding materials, which develop reactivity (Zhang et al., 2008). The boundless capabilities generated by nanotechnology have spurred significant interest in various fields, including power generation, electronics, and medicine, resulting in speedy modifications and creating new fields of study (Zhang et al., 2007). Whereas the effects of nanomedicine are endless, with the opportunity of modifying the diagnosis, imaging, and treatment of a disease, the toxicity of these particles has not been sufficiently examined in expressions of the increased risk rate of these particles and environmental effects. NPs can be swallowed, injected, inhaled, and absorbed through the skin. As such, the increased use of NPs in

manufacturing and industrial settings leads to an increase in the exposure rate. Therefore, risks and effects of NPs on human cells need further study (Oberdörster et al., 2005). An understanding of the genotoxic effect of special NPs on living cells and the mechanisms of this toxicity is significant when reducing a bulk compound to the nano-scale.

1.7.1 Nanoparticle applications

1.7.1.1 Nanoparticle applications in medicine

Nanotechnology has contributed significantly to the development of cancer treatment drugs due to the particles' ability to pass through typically resistant barriers, for example, the blood–brain barrier and tumour vesicles. The expansion of the surface area of the particles improves solubility with a high level of dissolution, which leads to increased bioavailability and enhanced therapeutic action (Chan, 2006). Damm et al. (2008) stated that the antimicrobial effectiveness of silver NPs was more effective than that of bulk-sized silver. The quantity of silver ions discharged from NPs was assessed and found to be more than some bulk particles, which is related to the large surface area of NPs. For this reason, silver nanoparticles are active in wound and infection treatment (Damm et al., 2008). Recently, nanoparticles have been used in gene treatments, biochemical imaging, drug delivery, protein detection, and tissue engineering. The change to the NP form allows the particles to act as a platform for the accumulation of several functions and constructions that can be used in drug targeting, recognition, and imaging. The shape and size of NPs can be reformed by the chosen drug loading and can affect the cellular intake and protection of drugs. Nano technology can deliver drugs more efficiently and effectively than

current techniques, and the improved pharmacokinetics increase patient observation and drug shelf life. Changing the size is responsible for releasing wavelengths that are produced from semiconductor and metallic nanostructures that facilitate biomedical imaging (Doane and Burda, 2012; Parveen et al., 2012). The surface area of NPs has specific characteristics that make them the best delivery vectors to improve the adsorption, concentration, and protection abilities of DNA and RNA (Sun et al., 2014).

1.7.2 Size-dependent toxicity

Once the size is reduced from the bulk form to the nano form, chemical and physical properties are changed such as surface area and immunogenicity were improved. For this reason, NPs are more active compared to the bulk form in term of reducing DNA damage with an increase in their fundamental characteristics that change their biological influences (Chen et al., 2009) Toxicological researchers have confirmed the increased toxicity of nanoparticles (<100 nm) compared to bulk form particles of the same chemical, raising interest in the effect of nanoparticles on human health. Several symptoms, including changes in hair colour, fatigue, loss of appetite, and weight have been detected in mice treated with gold NPs 8-37 nm. These symptoms were witnessed as a result of damage in the lung, liver, and spleen cells. After completing a 21-day course, all the treated mice died. Using genotoxic doses of NP forms, 50–100 nm sizes were found to be nontoxic in mice as these doses allow the production of an adequate antibody response and regulate the influence of diffusion. Toxic levels were observed when the surface genotoxic NPs were modified to 8-37 nm. The toxicity displayed by the genotoxic NPs was due to their failure to stimulate an immunological response, allowing the NPs to diffuse freely into the cells (Chen et

al., 2009). Evidence of toxicity was exposed when copper oxide (CuO) NPs were used to identify acute and chronic toxicity in Daphnia magna. Copper oxide NPs were ten times as toxic as micro particles, with the same result in *Vibrio fischeri* (Rossetto et al., 2014). Furthermore, many signs and symptoms of toxicity, such as epithelioid granulomas, necrosis, bronchial inflammation, and intestinal inflammation have occurred in mice treated with doses of carbon nanotubes; treating the mice with carbon bulk lowered the toxic effect. The effect of the carbon nanotubes was approximately the same when compared with a positive control (Lam et al., 2004). In conclusion, NPs are not always more toxic than micrometre particles, but the high toxicity levels of NPs raise specific concerns.

1.7.3 NP toxicity

Conflicting results have been reported in (2013) by Magdolenova et al in terms of explaining Nano form genotoxicity, because the mechanisms of toxicity depend on the physicochemical characteristics of Nano form that involve, dimensions, structure, crystal-like, solvation, external area of Nano form, also possibility of accumulation. In recent years, the mechanism of NP toxicity has been studied extensively by Magdolenova et al. (2013) and Manke et al. (2013). These researchers found that NPs can have direct or indirect effects on living cells, including primary or secondary genotoxicity, and the produce inhibition of the cell cycle, apoptosis, inflammation and reactive oxygen species (ROS).

1.7.3.1 Primary genotoxicity of NPs

Direct primary genotoxicity might operate by different mechanisms, such as the action of ROS, generated from reactive particle surfaces, or DNA-adduct formation, generated by reactive metabolites of a particle-associated organic compound. When

NPs infiltrate in the nucleus, they can interact with DNA replication, transcription, and repair. This interaction can be a mechanical interference or chemical binding to DNA molecules, which cause DNA instability and structural damage. In the mitosis phase of cell division, NPs can create acentric chromosome fragments or chromosomes alteration and inhibit the progress of mitosis (Magdolenova et al., 2013). Li et al. (2013) concluded that the association between NPs with a high binding to DNA and the strong inhibition of DNA replication, whereas NPs with less affinity had minimal or no effects on DNA replication. It has also been observed that hematite NPs can bind to DNA and modify its structure, and zinc and silver oxide (ZnO) NPs can affect the DNA form the effect can produce a specific protein, which is necessary for transformation, transcription, or mending, which are unable to penetrate the genetic material bound locations because the nano form controls the locations straight or by changing the structure of the DNA by alternate location relationships (Magdolenova et al., 2013).

1.7.3.2 Secondary genotoxicity

Genotoxicity produced by NPs does not require the NPs to be in continued direct contact with DNA; toxicity can be produced indirectly. Indirect primary genotoxicity can arise during many different mechanisms, such as interaction with nuclear proteins, disruption of the cell cycle, ROS generation, and the inhibition of antioxidant action (Magdolenova et al., 2013). Schins (2002) noted that secondary genotoxicity is related to an NP's dosage and length of exposure. NPs have been shown to produce reactive nitrogen species (RNS) and ROS in phagocytes through the beginning of DNA repair mechanisms and inflammation (Schins, 2013). In previous research, changes in cytokine levels were a product of an inflammatory

reaction in the peripheral blood of mice when treated with titanium dioxide NPs. The perused of inflammatory mediators increased phagocyte action to eradicate the NPs producing the genotoxicity. Proinflammatory gene transcription factors, such as mitogen-activated protein kinases, nuclear factor kappa B, and activator protein-1, were stimulated in response to NP treatment (Trouiller et al., 2009; Donaldson et al., 2012). When silica nanoparticles were used to treat mice and the RAW264.7 cell line, tumour necrosis factor (TNF)-α and levels of interleukin (IL)-1β and nitric oxide increased. This effect has an association with an amplified mRNA expression of inflammatory genes from macrophages with nitric oxide, IL-6, cyclooxygenase-2, and TNF-α. Treatment of the RAW264.7 cell line demonstrated ROS generation with a low expression of the antioxidant glutathione (GSH). Furthermore, Park and Park (2009) found that the generation of ROS contributes significantly to genotoxicity, and NPs that produce ROS can trigger the expression of proinflammatory mediators and stimulate immune cells.

1.7.4 Toxic NP-mediated oxidative stress

Oxidative stress is produced as a normal product of aerobic metabolism and can result in damage; oxidative stress is increased in some pathophysiological disorders. Antioxidant expression is stimulated via a number of methods. A direct correlation between ROS levels and oxidative stress is produced under NP toxicity. Furthermore, high levels of ROS generation or reduced levels of cellular antioxidants can lead to oxidative stress formation (Manke et al., 2013). NPs' size and surface area are thought to be connected to ROS. Li et al. (2008) reported that stimulated ROS formation was connected to the dissolution of NPs, changes to the surface of the metal, and the release of metal ions. Akhtar et al. (2012) demonstrated that ROS

formation, which can be exogenous or endogenous, is the product of free radicals, including hydroxyl radicals and superoxide anions, which damage DNA and other cellular structures. ROS has an effect on DNA and may lead to strand breaks or oxidised base lesions leading to cell death or mutation (Magdolenova et al., 2013). Free radicals cause electron chain dysfunction and membrane damage, which induces mitochondrial apoptosis because NPs can stop catalysis and the transmission of electrons to molecular oxygen or the electron transport chain of mitochondria (Manke et al., 2013). A relationship was found between ROS generation and cellular death after the treatment of HepG2 cells with silica NPs. Protein and mRNA levels of apoptotic genes p53, Bax, and caspase-3 were raised after exposure to silica NPs, and expression of the antioxidant GSH and the anti-apoptotic gene bcl-2 decreased, causing apoptosis (Ahmad et al.,2012). The transformations motivated by silica NPs were weakened, and treatment with the vitamin C scavenger ROS and increased cell viability, which shows ROS is responsible for medicinal cytotoxicity. Furthermore, there is a relationship between the disruption of cellular calcium homeostasis and NP-mediated ROS generation. Calcium regulates cellular metabolism, gene expression, and signal transaction; consequently, intracellular calcium levels are firmly adjusted, with an increase in intracellular calcium leading to metabolic imbalances, cellular dysfunction, and cell death. Levels of intracellular calcium increased with ZnO NP treatment, which caused cell death. This influence was somewhat inhibited by treatment with the antioxidant N-acetylcysteine, and cytotoxicity was completely reduced, which demonstrated the effect of oxidative stress on calcium homeostasis (Huang et al., 2010).

1.8 Reactive oxygen species (ROS)

ROS have received considerable attention in genotoxicology studies. ROS can be defined as molecules that can discharge oxygen atoms. Basically, they can be present in various concentrations as picomolar or micromolar in natural metabolism (Burns et al., 2012). Canonica et al. (2005) explained that hydrogen peroxide, O_2, OH, OOH, CO_3, and environmental systems have the ability to oxidise a range of biomolecules. Oxidative stress could cause cancer due to DNA, protein, or membrane lipid damage; for this reason, ROS have received significant attention in genotoxicology studies (Kumar et al., 2014). Bartosz (2006) reported that photolysis, as well as energy and electron transfer reactions, are responsible for ROS production. The reduction of oxygen can create free superoxide and hydroxyl radicals (Gutteridge, 1994). The activity of certain enzymes, such as xanthine oxidase and NADPH oxidase, leads to the production of the superoxide radical (. O-2). Dohi et al. (2010) demonstrated that superoxide (. O-2) is the original source of free oxygen radicals that work as oxidants or reductants (Dohi et al., 2010). High reactivity can be caused by the superoxide radical that is created by the organic more agents than any (. O-2) produced in interior biological processes where this might cause substantial DNA damage (Gutteridge, 1994). The active form of H_2O_2 could originate from the enzymatic processes of the superoxide. The combination of (. O-2) and H_2O_2 produce the hydroxyl radical (. OH) (Ben Shaul et al., 2001; Sewerynek et al., 1996). The hydroxyl radical is the name of the oxygen radical in chemistry; it has a large chance to cause biological damage when it becomes in direct interaction with all biological molecules. The hydroxyl radical can easily damage pyrimidine, purine bases, and other DNA structures (Gutteridge, 1994).

Furthermore, reactive oxygen species can cause very aggressive mitochondrial DNA damage because it is more sensitive than nuclear DNA (kumar et al., 2013).

1.9 Techniques used to measure genotoxicity

1.9.1 The Comet assay on lymphocyte

The Comet assay is a reliable and relatively uncomplicated technique used to estimate genotoxicity from DNA damage and repair. It was developed by Ostling and Johanson in 1984. Two different types of comet assays are used, depending on the type of DNA strand breakage. The first one is the neutral comet assay, which essentially determines double-strand DNA breakage. The second one is the alkaline comet assay, which detects single-strand damage and alkaline labile sites (Collins, 2004). However, the neutral type limits the potential of the assay. In 1988, Singh et al. further developed the Comet assay by using electrophoresis in alkaline conditions (pH >13). The alkaline method increases DNA migration and the sensitivity of the assay, which is useful in analysing genotoxic materials. Increasing DNA migration generates increased levels of partial excision repair single-strand breaks (SSBs) and double-strand breaks (DSBs), and as a result, alkaline-labile sites change to SSBs. The SSBs are quickly repaired and not measured as a mutagenic lesion; consequently, a high number of breaks found via the Comet assay could show high levels of damage (Collins, 2004). Treating cells with lesion-specific repair endonucleases helps determine the specific repair mechanism. Genotoxicity studies using Comet assays have compared the unscheduled DNA synthesis assay and the alkaline elution assay. The alkaline elution assay detects low levels of DNA damage, and generally a small sample is needed to perform the assay; it is also a sensitive technique with uncomplicated procedures, and solutions are easy to prepare (Tice et al., 2000).

This technique is extremely sensitive in evaluating SSBs, DSBs, and alkaline labile sites, but it is incapable of efficiently detecting DNA crosslinks. DNA crosslinks have a special ability to stabilise DNA and prevent its migration (Brendler-Schwaab et al., 2005). DNA crosslinks are important in mutagenesis; therefore, these cases will need more investigation in the form of extending the electrophoresis time or treating controls and treated samples with added genotoxic materials to compare DNA migration with and without the genotoxic effect (Tice et al., 2000). It has been shown that the adjusted comet protocol, although it can detect DNA–protein crosslinks, is not well suited for estimating DNA–DNA crosslinks and causes of reduced genotoxicity (Merk and Speit, 1999). Comet assays are also commonly used in molecular epidemiology to determine oxidative stress and carcinogenesis in human subjects, but different lifestyles should be accounted for as confounding variables when evaluating data as physical exercise and advanced age can induce an increase in DNA migration (Hartmann et al., 1994; Collins et al., 1997a' Anderson et al., 1998).

Comet assay on sperm

Spermatogenic cells are very sensitive and have a higher mutation rate than somatic cells (Walter et al., 1998; Winn et al., 2000). Moreover, Muriel et al. (2004) found that sperm chromatin has nine-times more single strand segments, and sperm nuclei contain more than double the rate of SSB compared to lymphocytes. Two different types of cells are found in the human body, diploid cells, which contain double the number of chromosomes, and haploid cells, which include sperm and ova and have half the number of chromosomes (World Health Organization, 2000; Walter et al., 1998). In the current study, the effect of flavonoids, such as quercetin, on both cell

types is applicable. In the alkaline sperm comet protocol, two lysis buffers are used: lysis 1 containing DL-dithiothreitol and lysis 2 with proteinase k from tritiracchium album. Two lysis buffers are freshly prepared and incubated for one hour.

1.9.2 Micronucleus assay

Fenech (2007) pointed out that a micronucleus (MN) is a small extra nuclear body that can be shaped throughout the anaphase of nuclear division. This small extra nuclear body cannot join into one of the daughter nuclei. Scientists first described micronuclei in red blood cells and called them Howell–Jolly bodies (Kirsch-Volders et al., 2003). The MN assay is classified as a preferred technique for evaluating DNA damage because it provides an accurate indication of chromosome mutations and breakage that are an expressed as a result of DNA damage, which is a substantial incident in carcinogenesis. Fenech (2000) illustrated that the shape of the micronucleus can be measured in one divided binucleated cell in humans and other mammalian cells by using an inhibitor substance (cytochalasin-B) to block the cytokinesis stage. Once cytochalasin B was applied in the MN assay, the name of the technique changed to the cytokinesis-block micronucleus assay (CBMN); this technique is the preferred method to measure micronuclei because micronucleus recurrences are changeable with repeated divisions and cannot be recognised in a population of dividing cells (Fenech, 1997). DNA damage, gene amplification, loss of chromosomes, inhibition of cell division, excision repair, necrosis, and apoptosis can be easily detected efficiently by CBMN (Fenech, 2006; Fenech et al., 2011). The CBMN assay can be used to easily recognise and score micronuclei in cells that have completed nuclear division. The cells can be used for the recognition of

micronuclei, nuclear buds (NBUDs), and nucleoplasmic bridges (NPBs)

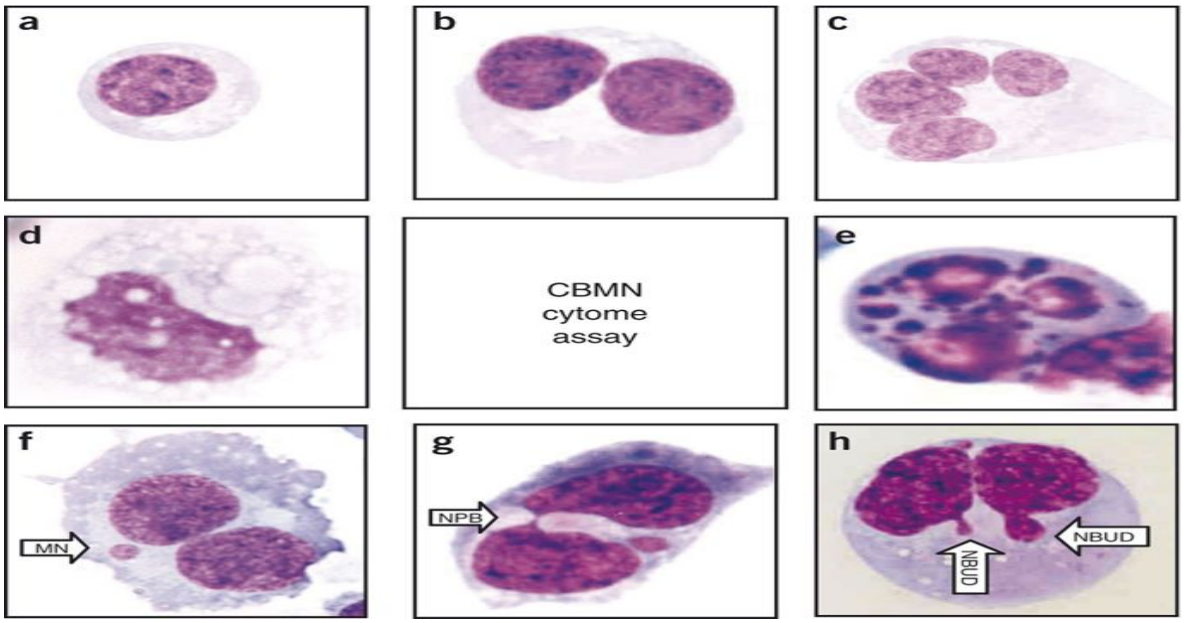


Figure 9 : The cells scored in the CBMN assay. (a) mononucleated (MonoNC cell); (b) binucleated (BiNC) cell; (c) multinucleated (multiNC) cell; (d) early necrotic cell; (e) late apoptotic cell; (f) BN cell with one or more MNi; (g) BN with an NPB and a MN; (h) BN cell with NBUDs (Fenech, 2007).

Advantages of the CBMN technique are that it is a relatively quick process and has a reasonable cost compared to other cytogenetic methods (Fenech, 2000; Fenech, 2007).

1.9.3 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) uses the capability of DNA to create another strand opposite of the exhibited template strand. Saiki et al. (1988) indicated that the amplification of a few copies or at least a single copy of DNA proceeds through some preparations of magnitude. The preparations, which create more than one million specific DNA sequences, need a primer that accelerates the DNA sequence by adding a nucleotide. The primers are considered the specific fragment of the template sequence that the detector is requested to amplify (Saiki et al., 1988). In 1993, the American biochemist Kary Mullis was awarded a Nobel Prize for developing modified PCR in 1984, although Gobind Khorana was the first to describe the basic principles of amplifying a part of DNA using primers in 1971 (Joshi and Deshpande, 2011).

The PCR technique is often used in molecular biology and medical research because it has many advantages; for example, small samples can be used, chemicals and primer are priced reasonably, its procedures and steps are easy and understandable, and an unlimited number of copies of any DNA sequence can be synthesised. Additionally, the PCR can amplify a small DNA fragment even if the fragment of the DNA is of poor quality (Joshi and Deshpande, 2011). This technique has several uses in many fields, such as forensic medicine, which is using the PCR to confirm the paternity from genetic fingerprints. Moreover, PCR can be used to diagnose infectious diseases, such as hepatitis and human immunodeficiency virus, as well as *Mycobacterium tuberculosis* via qualitative PCR. Bartlett and Stirling (2003) reported that PCR can be used for DNA cloning, genetic mapping, mutation generation, and gene expression. In this study, the PCR technique was used to

investigate gene expressions connected with the inflammatory pathway, such as COX1 and COX2, as well as p53 and Bcl-2, which are related to apoptosis. Lindahl (1993) pointed out that PCR cannot differentiate between a DNA sequence collected from a living cell and DNA obtained from a dead cell.

Another technique, called real-time PCR (RT-PCR), can be used for gene expression when only a small sample of DNA, cDNA, or RNA (PCR products) is available. The RT-PCR protocol depends on the determination of fluorescence made by the cumulative target molecules. This fluorescent target molecule has dyes that link to the sequence-specific probes or DNA strands. RT-PCR is commonly used because of the accuracy of results and the quickness and sensitivity of the method. Another advantage of RT-PCR is its low rate of contamination due to PCR products (i.e., cDNA or RNA) (Klein, 2002). Wong and Medrano (2005) reported that experiments using RT-PCR require a full understanding of the technique because it is a very sensitive procedure. PCR is an effective technique commonly used in genotoxicity and gene expression studies.

1.9.4 Western blots (WB)

Western blotting (WB) is a powerful technique and the most common method used to examine the presence of a specific protein in extracted complex cells. After 1979, there was a huge development of protein blotting (Kurien, 2006). WB can be described as comprising five main steps: (1) the target proteins are diffused through polyacrylamide gel electrophoresis (SDS-gel); (2) the proteins are transferred and repositioned from the SDS-gel to an artificial membrane made from nitrocellulose and polyvinylidene fluoride PVDF (electroelution); (3) the specific primary antibody is used to fix the protein attached to the membrane; (4) the secondary antibody is

incubated to bind the protein-primary antibody complex, for example, horseradish peroxidase (HRP); and (5) the detected protein bands can be imaged using an ECL substrate and dye (Liu et al., 2014; Mahmood and Yang, 2012). Westermeier and Marouga (2005) stated that compared to other immunoassay techniques, WB is an ideal technique for measuring protein expression because it produces easy-to-understand results, is accurate, and has a reasonable cost. It is generally a reliable technique and provides additional information through the principal steps. When the results are not well-matched, there are marks regarding what should be investigated to determine the potential error: the band could have a higher or lower molecular weight than supposed, or it could be a poly band instead of a single band. A small band shows that the protein has been fragmented or destroyed, whereas a band that occurs at a higher level could reflect an enlargement in the protein size. This refers to the formation of glycosylation or multimeter and the accumulation of amino acids found in proteins. This technique also has the capability to display the presence of a particular protein by size or during the binding of an antibody, which confirms the capability of the method to monitor protein fractions through the protein purification procedure. This is useful for comparing protein expression from various cells or the specific proteins that respond to drug treatment.

Dechend et al. (2006) reported that in the Western blot experiments; there is a particular reaction between an antigen and an antibody. The antigen is usually a peptide or a protein, and it is detected by the antibody. The important point of the reaction is between the sites found on the arms of the antibody and small parts of the antigen (epitope). Kurien and Scofield (2006) explained that two categories of the antibody are used in the Western blot test: the polyclonal antibody and the

monoclonal antibody. The polyclonal antibodies are prepared by injecting a whole antigen into the animal body to activate the immune system to create specific antibodies against that antigen, whereas monoclonal antibodies can be prepared when injecting only epitopes, which are the main part of the antigen to the animal body. This method yields monoclonal antibodies that bind to single epitopes, while polyclonal antibodies can bind to different epitopes.

The Western blotting technique has been commonly used recently to evaluate the genotoxicity of nanoparticles. Kumar et al. (2014) investigated protein expression using the effect of ZnO-engineered NPs in lymphocyte cells from healthy donors and chronic obstructive pulmonary disease, lung cancer, and asthma patients. They found significant results ($P<0.05$) showing a rise in the protein expression of tumour suppressor proteins p53 and p21, in all except the asthma patients (Kumar et al., 2014). Furthermore, the expression of Fanconi anaemia and connection of the breast cancer pathway to genes was confirmed by the Western blot test when the effect of PJ34 was studied. Also, the genotoxicity of cells was induced by melphalan in human multiple myeloma cells. The apoptosis and cell cycle phases were scored by flow cytometry, and no significant result of PJ34 and melphalan on cell proliferation were determined (Xiong et al., 2015). Genotoxicity and cytotoxicity were studied in breast cancer cell lines (MCF-7 and MCF-10A) using 4-hydroxytamoxifen with Malaysian Tualang honey; the effect on cellular DNA damage was detected by the Comet assay, and the expression of DNA repair enzymes was examined using Western blotting. The results indicated that 4-hydroxytamoxifen was cytotoxic to both cell lines, whereas Tualang honey was only cytotoxic to the MCF-7 cells; it significantly decreased the cytotoxicity effect of 4-hydroxytamoxifen in MCF-10A

cells but not in MCF-7 cells. Tualang honey has the capability to improve the proliferation of MCF-10A and inhibit growth caused by 4-hydroxytamoxifen. Tualang honey can protect cancer cells from DNA damage caused by increased levels of 4-hydroxytamoxifen, which is how it decreases the genotoxic effect of 4-hydroxytamoxifen on non-cancerous cells. This result was supported by the elevated expression levels of DNA repair proteins Ku70 and Ku80 in MCF-10A cells of Tualang honey. The results show that Tualang honey can improve the effectiveness of the DNA repair mechanism in these cells (Yaacob and Ismail, 2014).

Aims and Objectives

This study aims to investigate the geno protective effect of the bulk and nanoparticle (NP) forms of the antioxidant compound quercetin; in lymphocytes from TB patients, to reduce the spread of the disease that become prevalent again in the UK due to the migrants.

Objectives: Quercetin will be used to treat ex-vivo lymphocytes and sperm. DNA damage will be estimated using the Comet assay; the results will be confirmed by micronucleus assay in peripheral blood lymphocytes collected from tuberculosis patients and the results will be compared with data collected from healthy individuals (i.e., lymphocytes and sperm). The influence of two forms of quercetin on diploid and haploid cells will be compared; as lymphocytes are diploid cells with two sets of chromosomes, and spermatozoa are haploid cells with one set of chromosomes, both of these will be tested using three different concentrations of quercetin. The Comet repair assay's usefulness in monitoring DNA damage repair will be studied by treating lymphocytes with PhIP and IQ, a DNA damaging food mutagen and analysing DNA repair in the presence and absence of both forms of quercetin. The

role of bulk and nano forms of quercetin on catalase protein expression will be studied using the WB technique to analyse lymphocytes from patients with TB and compare the results with lymphocytes from healthy individuals. Finally, the gene expression of cyclooxygenase enzymes that show an inflammatory pathway, such as COX1 and COX2, will be examined, as well as p53 and bcl-2, which reflect apoptosis. Lymphocytes collected from patients with TB will be compared with lymphocytes collected from healthy individuals via real-time PCR.

2 Chapter (2) Materials and Methods

2.1 Materials and Methods

2.2 Materials

List of reagents and chemicals which are used in this study illustrated in the following tables, CAS number and provider.

Chemicals	CAS number	Provider
2-amino-1methyl-6-phenylimidazo[4,5-b] pyridine (PhIP)	105650-23-5	Toronto Research Chemicals, Toronto Canada
2-amino-3-methylidazo[4,5-f] quinoline (IQ)	76180-96-6	Toronto Research Chemicals, Toronto Canada
Agarose, low gelling temperature	39346-81-1	Fisher scientific
Albumin, Bovine serum, Fraction V, minimum 96% lyophilized power	9048-46-8	Sigma, UK
Bis-acrylamide,30% solution	110-26-9.	Sigma, UK
Bleomycin sulfate	9041-93-4	Sigma UK
Bradford Protein Assay Kit 1	9015-82-1	Bio-Rad
Corning® cell culture flasks		Sigma-Aldrich
Cytochalasin-B	14930-96-2	Sigma-Aldrich UK
DL-Dithiothreitol	3483-12-3	Sigma-Aldrich UK
DMSO	67-68-5	BDH, UK
Ethanol	64-17-5	Sigma, UK
Ethidium bromide	1239-45-8	Sigma, UK
Fast SYBR® Green Master Mix		Life Technologies
Foetal Bovine Serum	9014-81-7	GIBCO Invitrogen UK
Hs ACT 1 SG Quanti Tec Primer Assay		Qiagen
Hydrogen peroxide	7722-84-1	Sigma, UK
iScript ™ c DNA Synthesis Kit		Biorad
Low melting point (NMP) agarose	39346-81-1	Invitrogen, UK
Lymphoprep	26873-85-8	Stem cell Technology

MicroAmp® Optical 8-Cap Strips	7732-18-5	Qiagen
Mitomycin C	50-07-7	Sigma-Aldrich UK
MTT cell proliferation kit	61825-94-3	Invitrogen, UK
Na2EDTA·2H2O	6381-92-6	Sigma, UK
NaCl	7647-14-5	Sigma, UK
NaOH BDH	1310-73-2	BDH, UK
Normal melting point (NMP)agarose	9012-36-6	Invitrogen, UK
Nucleus-Free Water	7732-18-5	Promega
PBS phosphate buffered saline	n/a	Sigma, UK
PCR max Eco 48 pates Pk/50	n/a	Scientific laboratory supplier
PCR max Eco 48 pates seals Pk/50	n/a	Scientific laboratory supplier
Penicillin-Streptomycin	P4333-100ML	Sigma-Aldrich
Phytohaemagglutinin-M (PHA-M)	L2646-10MG	Sigma-Aldrich
Precision Plus Protein™ Dual color Standards	161-0374	Bio-Rad
Protein Kinase	141436-78-4	Sigma, UK
QIAamp® RNA Blood Mini Kit ID 52304	n/a	Qiagen
Reverse Transcription System	9068-38-6	Promega
RPMI-1640 Medium, GlutaMAX™	251310-57-3	Thermo Fisher Scientific
Thermo Scientific Microscopic slides	n/a	Fisher scientific
Thick Blot Filter Paper 7.5x10cm	n/a	Bio-Rad
Triton X-100	9002-93-1	Sigma, UK
Trizma Base	77-86-1	Sigma, UK
Trypan blue	72-57-1	Sigma, UK
Ultra-pure Agarose	9012-36-6	Fisher scientific
Western ECL Substrate, 200 ml	n/a	Bio-Rad

Table 1 : Chemicals and reagent with their CAS numbers, and supplier.

Primer	Identification number	Provider
COX-1 Primer	QT00210280	Qiagen
COX-2 Primer	QT00040586	Qiagen
Bcl-2 Primer	QT00025011	Qiagen
P53 Primer	QT00050785	Qiagen

Table 2 : Primers used in PCR technique.

Antibodies	Identification number	Provider
Anti-rabbit IgG	7074	Cell Signalling technology
Anti-Catalase antibody[EPR20198]	Ab209211	Abcam
Anti-GAPDH antibody [EPR16891]	Ab181602	Abcam

Table 3: Antibodies used in WB technique.

2.3 Equipment

Equipment	Provider
Cell culture flasks	Corning, Fisher Scientific, UK
Centrifuge Mistral 3000	MSE, gmi, Albertville,USA
Coplin jar	VWR, UK
Cover glass	VWR,UK
Coverslips	Strutted, Germany
Dry incubator LKBBIOCHROM	Leec LTD, Nottingham, UK
Electrophoresis power supply	Consort(E861),Belgium
Electrophoresis tank(Hu20)	SciePlas, Renfrewshire, UK
Slides thermo	VWR,UK
Tubes (Eppendorf)	Sigma,UK

Incubator	Flow Labs, UK
Freezer -20and -80°C	Sanyo,Ultra low,Japan
Fume hood	Maich-Aire,Bolton,uk
Heparinised vacutainers	Geiner-Bio-one ,Germany
Ice Machine (ScotsmanAF100)	Namur, Belgium
Comet 4 imaging software	Kinetic imaging , Liverpool, UK
Microscope (light)	Nikon
Liquid nitrogen Dewar	Biostar,UK
Small centrifuge	MSE,gmi,Albertville,USA
Haemocytometer	Sigma-Aldrich
pH metre	Dummow,uk
pipette 1,5,10,20,50,100.500.1000µl	Gilson, USA
Sterile tubes 15,30,50ml	BD Swindo,UK
Water bath	Grant instruments,UK
Implen nano-photometer P330	Implen Gmbh. Munich. Germany
ECO 48 PCR Machin	SLS (scientific laboratory supplier). UK
Western blots Electrophoresis tank	Bio Rad. USA
MRX-Microplate reader CXD3263	DYNEX-Technologies USA
Western blots box imaging	Syngene. UK

Table 4 : Equipment used in this project.

2.4 Questionnaire

The donors who donated sperm or blood both healthy and patients were required to complete a consent form and answer some relevant questions.The questions were about smoking, alchol, age, lifestyle, hereditary factors, drugs, diet and use of vitamin supplements.

2.5 Blood and sperm samples collection

Sperm samples were collected from 20 healthy non-smoking donors after two days abstinence. Samples were analyzed immediately based on WHO criteria. Motility, viscosity, sample volume, sperm count and pH were evaluated. If all parameters were within the normal value the rest of the samples were divided into 100µl subsamples in small Eppendorf® tubes and dipped in liquid nitrogen. These 100µl of semen subsamples were stored in a freezer at – 80 °C and used in the Comet assay.

Blood was collected using venepuncture blood collection set which was labelled lithium heparin coated tubes from both healthy non-smoking volunteers and (TB) patients who had completed and signed the consent form by a qualified phlebotomist. Blood was diluted 1:1 with RPMI-1640 medium and then DMSO is added to 10% from the total volume, then samples were subdivided into 1ml in each Eppendorf® and stored at -80°C. However, fresh blood samples which were not diluted were used in the micronucleus assay, qPCR, and Western blot techniques. Patient's blood was collected from St.Luke's Hospital in Bradford, West Yorkshire, UK. All patient's donated 10-20 ml of blood, the blood samples were kept at room temperature for a maximum of 48 hours while waiting for use.

2.6 Ethical considerations

The laboratory received ethical approval from the University of Bradford (reference number 0405/8), which was accepted by the Research Support & the Governance Office Bradford Teaching Hospital NHS Foundation ReDA number 1202 and Leeds East local Ethics Committee (Reference number 12/YH/0464). The information was obtained by questionnaire, and patient details were coded, and given an allocated number.

2.7 Preparation of quercetin nano particles

The preparation of a nanoform of quercitin involved dissolving bulk quercitin in ethanol to prepare a concentration of 1mg/ml and 5ml from this preparation was injected through one side of the channel of Microfluidic reactors (Y shape) shown in figure 10 which was used to prepare a nano form of quercetin. The other side of Microfluidic reactors were used to inject 20 ml of water at the rate of 15 ml/hr and 60 ml/hr respectively. The production was collected in a beaker of 50ml capacity containing 0.5 % w/w HPMV and PVP and 1% SLS on the magnetic stirrer. The magnetic stirrer was used to mix the component together. The mixture was left on the stirrer for 10 min. Finally the Malvern nano sizer DLS was used to measure the particles size (Figure 11).

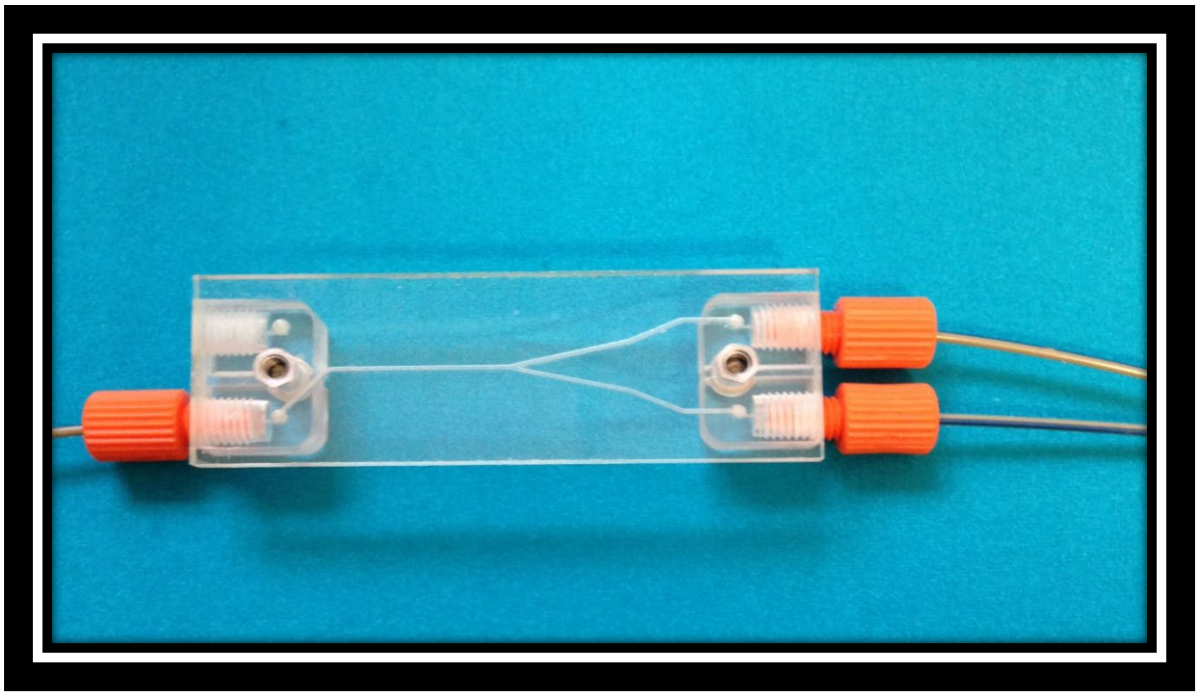


Figure 10 : Microfluidic Y shape used to prepare nano form of quercetin.

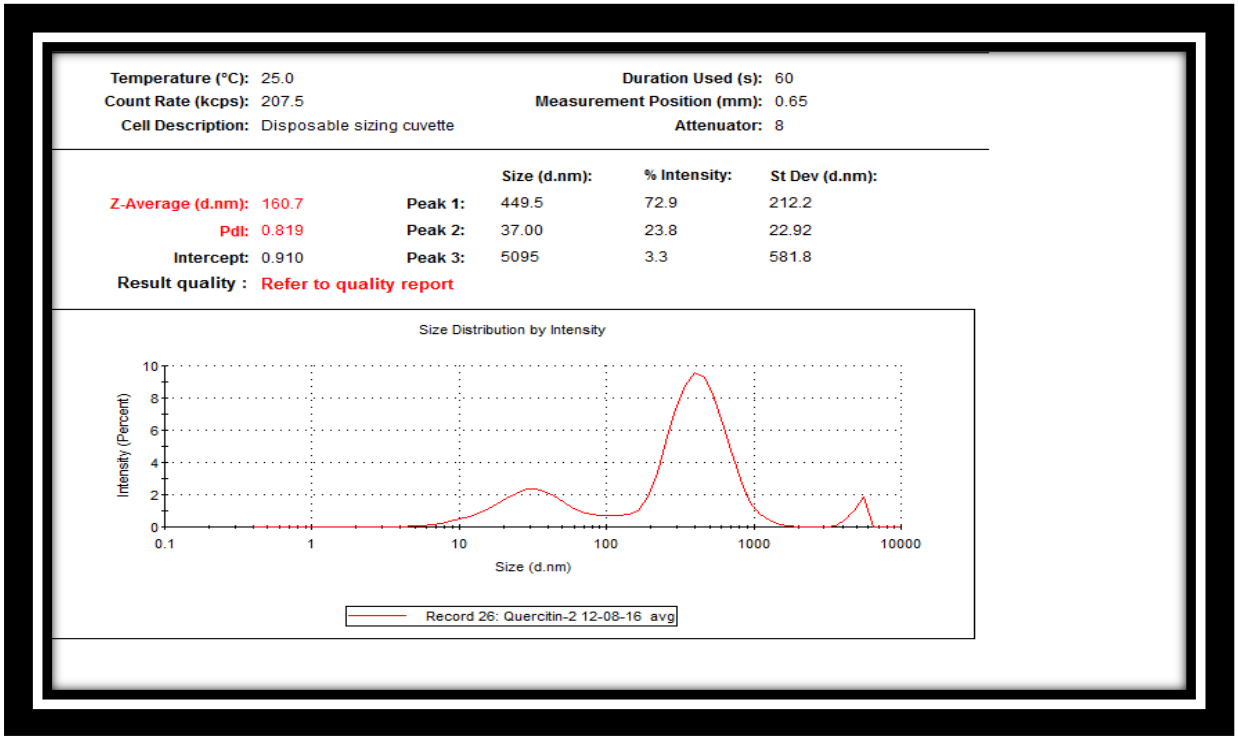


Figure 11 : Size Distribution by intensity of quercetin nano using Malvern nano sizer.

2.8 Quercetin concentrations

Three concentrations of quercetin were used in this project for both sizes bulk and nano forms. The concentrations that were used are 10µM, 25µM, 100µM. These concentrations were chosen based on the previous articles that were published by (Cemeli et al., 2004), (Najafzadeh et al., 2009) and (Habas et al.,2018). The publications were considering three concentrations of quercetin were safely used with lymphocytes in vivo and vitro, whereas the concentration above 200 µM of quercetin both forms where neglected, they reported that high dose of quercetin is cytotoxic dose. Furthermore, quercetin is carcinogenic in male rats, inducing renal adenoma when fed at 2000 mg/kg (National toxicology Program Ed.,1-171,1979; Harwood et al., 2007).

2.9 Cell viability

Different quercetin concentrations (nano and bulk forms) 10 µM, 25 µM and 100µM were used to treat lymphocytes as usual. The tubes were centrifuged at 705g for five minutes at room temperature. After that, 0.4% of Trypan blue stain was added to treated cells 1:1 ratio. The lymphocytes were examined under a microscope after 30 minutes of treatment. A total of 100 cells from treated lymphocytes were scored per slide using an improved Neubauer counting chamber.

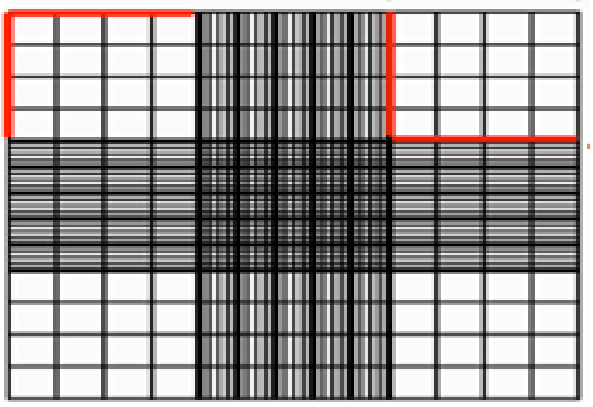


Figure 12 : IMPPROVED NEUBAUER counting chamber used for Cell viability.

The cells that were penetrated by Trypan blue and absorbed dark blue colour are indicative of cell cytotoxicity and cell death, whereas the cells with colourless cytoplasm represent the live cells. The proportion of the live to dead cells was used to determine the quercetin concentrations that could be used safely in the Comet assay.

2.10 MTT assay tetrazolium dye

The MTT assay was used to assess the cytotoxicity of quercetin concentrations in cultured lymphocytes. The MTT powder was prepared at a concentration of 5mg/ml. Lymphocytes were seeded 10,000-20,000 cells per well in a 96-well plate and incubated at 37 ˚C in a cell culture incubator overnight. The cells firmly attach to bottom surface during this time. The next day the medium were aspirated from each

well, and the treatment solution was added at the desired concentration. The treated cells were incubated at 37 ˚C for desired period of exposure. After the exposure time completed the medium were replaced by 90µl per well, and 10 µl of MTT day were added to each well and incubated for 4 hours. On completion of the incubation time 200 µl of DMSO were added to each well. Finally, absorbance was measured after 10 minutes at 550nm. The cell survival percentages were calculated from absorbance of drug concentrations divided by negative control absorbance and multiplied by100.

Ab of drug ÷ Ab of negative control x100= Cell survival ? %.

The results of this assay suggested that, the three quercetin concentrations both forms were showed the cell survival over 75 %.

2.11 Lymphocyte Comet assay

2.11.1 Treatment

Frozen lymphocytes were allowed to thaw at room temperature. Once the blood liquefied, 100µl were mixed in an Eppendorf® tube with 890 RPMI-1640 medium. The experiments were performed in six Eppendorf® tubes; the first tube contained untreated cells and excipient, the second tube was the positive control which contained blood, media, excipient and 10µl of 60µM hydrogen peroxide. The rest of the tubes were contained 10µl of H_2O_2 and three concentrations each of quercetin nano and bulk forms. The end volume of Eppendorf® tube was 1000µl.The incubation time for all Eppendorf's® 30 were minutes at 37°C. After incubation the tubes were centrifuged for 3 minutes at 705g. Next 900 µL of supernatant was removed from each Eppendorf® tube and 100µl of low melting point (0.5%) agarose was added to residual cells with gently mixing, 100µl from the mixture (cells and agarose) was spread on slides previously coated by 1% of normal melting agarose

(NMP). Then slides were covered by the cover slip and placed on a tray containing ice for 5 minutes for gelation.

2.11.2 Lysis

This step was performed by removing the cover slip and immersing the slides in a black box that contained freshly prepared lysis buffer; 2.5mM NaCl, 100mM EDTA, 10mM Trizma, 10% DMSO 1%Triton X-100. The solution was adjusted at pH 10. The slides stayed overnight in the lysing buffer at 2-4˚C This solution concentrated by salts and detergent can easily dissolve cell membranes and protein bonding, and the RNA bonds were lacerated.

2.11.3 Electrophoresis

After cell lysis, the electrophoresis tank was cleaned and filled with a fresh alkaline solution that consisted from10 ml of 200mM EDTA plus 60ml of 10M NaOH to allow an alkaline P^H >13. Slides were arranged horizontally in an electrophoresis buffer tank in the refrigerator for 30 minutes that was enough to unwind the DNA. Electrophoresis was performed in the refrigerator for 30 minutes at 300mA and 25 volts using a special power supply pack.

2.11.4 Neutralization

The neutralization buffer used in this step consisted of 0.4M Tris-HCl, pH 7.5. Slides were washed by this buffer three times for 5 minutes each time.

2.11.5 Staining and scoring

The stain which was used in this laboratory was ethidium bromide (20µg/ml), 60µl of this solution were added for each slide in the dark room. Slides were covered with a coverslip and scored after 10 minutes to avoid cell dehydration. A fluorescence microscope linked to a computer was used. The magnification used was 20x, and

100 cells from each slide were scored randomly, the data were transferred to another computer and analysed. Two parameters, %Tail DNA and Olive tail moment were considered for the Comet assay

2.11.6 Sperm comet assay

The procedure used for the sperm comet assay and lymphocyte comet assay were similar except for the steps listed below.

1) 2µl of sperm was added to Eppendorf® tubes in treatment stage.

2) Incubation was performed at 32°C for one hour in the water-bath.

3) Two lysis buffers were used in the sperm comet assay. First lysis buffer contained 77mg of DL-Dithiothreitol/50mL of the original lysis buffer. Second lysis buffer has 2.5mg of Protein Kinase /50mL of original lysis buffer.

4) Time for DNA unwinding in the refrigerator and electrophoresis was 20 minutes each. All these differences were because haploid sperm to be more sensitive than diploid lymphocytes.

2.12 Micronucleus assay

2.12.1 Preparation culture media

The preparation was carried out under strict sterile conditions in a laminar fume hood. The process was started with addition of 1% of Penicillin-streptomycin and 15% Foetal bovine serum to RPMI 1640 medium which contained 25mM Hepes buffer and L-Glutamine amino acid. Once the medium is prepared, 4.5ml was transferred to 25 cm^3 Corning cultures flasks. The flasks are ready to use and can be stored at -20°C for three months to use later.

2.12.2 Lymphocyte cultures

Corning culture flasks, 25 cm^3 in capacity, were incubated for 30 minutes at 37°C until medium was completely thawed. Then at zero hours, 500µl of fresh blood collected from healthy volunteers or TB patients was added to each flask, followed by 130µl of phytohaemagglutinin (PHA). 50µl of excipient (basic solution of drug components) was added to the untreated cells. 50µl of mitomycin C (0.4µM) was added as the positive control. Different concentrations of quercetin 10 µM, 25 µM, 100µM from both bulk and nano forms were added to the rest of the flasks. After that the flasks were incubated at 37°C for 24 hours. In total there were nine flasks. The following table illustrates the number of flasks and the chemicals that were used. Phytohaemagglutinin is used to induce the cultured lymphocytes for cell division. The micronuclei of divided binucleated cells (indicating cell division) are then measured to identify and evaluate DNA damage (chromosome breaks or loss). Nuclear-cytoplasmic buds (NPBs) specify DNA disrepair, and an amplified DNA repair complex can be confirmed by NBUDs and chromosome rearrangement. Chromosome losses or fragments increase the number of micronuclei (Fenech, 2000; Schmid, 1975). Asymmetrical chromosome rearrangement or DNA strand break disrepair can be detected by NPBs, which might be the reason for chromatids being transported to the opposite end of the cell (Thomas et al., 2003). Fenech (2002) and Serrano-Garcia and Montero (2001) demonstrated that NBUDs reflect the probability of gene amplification, which is considered to be evidence of genotoxic exposure. The CBMN technique is an efficient method, but its efficiency is dependent on the Cytochalasin-B concentration; then, the optimal dose should be adjusted for each cell type to confirm that no cells escape from the cytokinesis-block (Surrallés et al., 1992).

Flask Number	Media	Blood	PHA	Chemicals
Flask 1 NC	4.5 ml	500µl	100µl	50µl Excipient
Flask 2 PC	4.5 ml	500µl	100µl	50µl Mitomycin+50µl Excipient
Flask 3 B	4.5 ml	500µl	100µl	0.75 µg/ml Bleomycin +50µl Excipient
Flask 4	4.5 ml	500µl	100µl	10µl of quercetin bulk (10µM) + 0.75 µg/ml Bleomycin
Flask 5	4.5 ml	500µl	100µl	10µl of quercetin nano (10µM) + 0.75 µg/ml Bleomycin
Flask 6	4.5 ml	500µl	100µl	25µl of quercetin bulk (25µM) + 0.75 µg/ml Bleomycin
Flask 7	4.5 ml	500µl	100µl	25µl of quercetin nano (25µM) + 0.75 µg/ml Bleomycin
Flask 8	4.5 ml	500µl	100µl	100µl of quercetin bulk (100µM) + 0.75 µg/ml Bleomycin
Flask 9	4.5 ml	500µl	100µl	100µl of quercetin nano (100µM) + 0.75 µg/ml Bleomycin

Table 5 : Micronucleus experimental design and amount of chemicals

2.12.3 Blocking stage

Cytochalasin B is the chemical used to block the culture processes, 30µl of this chemical (6µg/ml) were added to each flask, after 44 hours of incubation. Cyto B blocks cytoplasmic division inhibiting formation of actin filaments, thus arresting the cells in the bi- nucleated form.

2.12.4 Hypotonic treatment

All the flasks were removed from incubation at 77 hours and contents transferred to separate falcon tubes. The falcon tubes were centrifuged at 107g for 8 min. The supernatants were removed using vacuum suction, leaving 500µl with the sediment of the cells. The cells were then -suspended again in 5ml of cold freshly prepared hypotonic solution (110Mm potassium chloride). Next; the tubes were incubated at 4

^{O}C for 15 min. The centrifugation was repeated with the same time and speed. The supernatant was removed, and the cells were re-suspended by gently shaking.

2.12.5 Cell fixation

A fixing solution that is called carneys solution (1 volume of glacial acetic acid and 3 volumes of ethanol) was prepared; also 38% formaldehyde is required. The solution was used three times to fix the cells.

2.12.5.1 First fixation

5 ml of carneys solution were added in each tube drop by drop, and the contents of the tubes were mixed by vortex machine followed by three drops of formaldehyde. The tubes were centrifuged 8 minutes at 107g. Subsequently, the supernatant was removed by vacuum suction leaving 500µl. Tubes were gently shaken to re-suspend cells again.

2.12.5.2 Second and third Fixation

Second and third fixations were the same at the first fixation, but without adding formaldehyde.

2.12.5.3 Staining of cells and scoring

After centrifugation, 100µl cell suspension was mixed with 300-600µl of carneys solution. 20-30µl of cell suspension was placed on clean and dry glass slide; two slides were made for each concentration to confirm the result. Subsequently, slides were kept drying overnight. The slides were then stained with 5% Giemsa stain (51 ml 0.2 M NaH_2PO_4 with 49 ml 0.2 M Na_2HPO_4) for 20 minutes. The slides were washed using tap water and dried. Once the slides were completely dry, they were fixed by rubber fix gum (DPX) and protected by a coverslip. 500 cells were scored for each slide to obtain 1000 cells for each tube. Scoring of micronuclei, nuclear

buds (NBUDs), nucleoplasmic bridges (NPBs), and chromosome rearrangement was performed to evaluate the cytogenetic damage.

2.12.6 The influence of heterocyclic amines PhIP and IQ on DNA in lymphocytes and sperm that were treated with different concentrations of quercetin

The concentrations 160μM and 140μM of 2-amino-3-methylidazo [4, 5-f] quinoline (IQ) and also 140μM and 100μM of 2-amino-1methyl-6-phenylimidazo [4,5-b] pyridine (PhIP) were used as genotoxic agents to induce DNA damage in lymphocytes and sperm. These were incubated for 20-30 minutes at 37°C before being embedded in 5% low melting point (LMP) agarose on slides pre-coated with agarose. The stock solutions of IQ and PhIP were prepared using deionized distilled water (ddH_2O) and stored in a freezer at 2-4°C. The oxidative DNA damage resulting from the food mutagens was evaluated by the Comet assay.

2.12.6.1 Cell treatment

Nine Eppendorf® tubes were selected and labelled to study the influence of both PhIP and IQ food mutagens on lymphocytes and sperm; the experiments were designed by untreated cells, positive control with 60 μM H_2O_2 in lymphocytes and 50 μM H_2O_2 in sperm, the third Eppendorf® tube was PhIP or IQ only and the rest of Eppendorf® were induced DNA damage using PhIP or IQ and treated with quercetin bulk and nano in same time.

The following table illustrates the experimental design.

Eppendorf Number	Media PRMI	Blood	Chemicals
Eppendorf 1 NC	890µl	100µl	10µl Excipient
Eppendorf 2 PC	880µl	100µ l	10µl Excipient +10µl (60 µMH_2O_2) IQ
Eppendorf 3 PC	880µl	100µ l	10µl Excipient +10µl (160 µM) IQ
Eppendorf 4	870µl	100µl	10µl Excipient +10µl of quercetin bulk (10µM) +10µl (160 µM) IQ
Eppendorf 5	870µl	100µl	10µl Excipient +10µl of quercetin nano (10µM) + 10µl (160 µM) IQ
Eppendorf 6	870µl	100µl	10µl Excipient +10µl of quercetin bulk (25µM) +10µl (160 µM) IQ
Eppendorf 7	870µl	100µl	10µl Excipient +10µl of quercetin nano (25µM) + 10µl (160 µM) IQ
Eppendorf 8	870µl	100µl	10µl Excipient +10µl of quercetin bulk (100µM) + 10µl (160 µM) IQ
Eppendorf 9	870µl	100µl	10µl Excipient +10µl of quercetin nano (100µM) + 10µl (160 µM) IQ

Table 6 : Experimental designs for comet assay were lymphocytes induced DNA damage by IQ or PhIP and treated nano and bulk forms of quercetin.

2.12.6.2 Mixing the cells with agarose

Once the incubation period was completed, all the tubes were centrifuged for 3 minutes at 110 g, after centrifugation 900µl supernatant was removed and the 100 µl of supernatant along with the cell pellet was mixed gently with 100 µl of LMP agarose. 100 µl each from the mixture was placed on slides pre-coated with NMP agarose and covered by a coverslip; two slides for each Eppendorf® tube were made to determine the results. The slides were kept on the ice surface for 5 minutes for gelation. This was followed by removal of coverslips transfer of slides into a black box containing cold lysis solution. The components of fresh cold lysis solution included 100 mM EDTA, 2.5 M NaCl, 10 mM Trizma base, 10% DMSO and 1% Triton X-100, pH 10. The reason for using lysis solution is to denature protein and lysis of cell membranes.

2.12.6.3 DNA electrophoresis

This step starts by unwinding DNA for lymphocytes or sperm. Slides were arranged in a horizontal position inside the electrophoresis tank; the slides were covered by an electrophoresis buffer which consisted of (300 mM NaOH and 1 mM EDTA, pH > 13). The cells were incubated for 30 minutes at 4°C for DNA unwinding, after that electrophoresis was operated for 30 minutes at a constant voltage of 25 V and 290-300 mA.

2.12.6.4 Neutralisation and scoring

Once the electrophoresis time was completed, the slides were washed by neutralisation buffer consisting of (0.4 M Tris-Hcl, pH 7.5) three times where each time takes 5 minutes. The ethidium bromides stain 20 µg/ml was used to stain the cells in the dark room and covered with cover-slips for 10 minutes and scoring. 100 cells were scored randomly for each slide by a fluorescence microscope linked to a CCD camera and Computer system (Andor Technology Ltd, Belfast, UK). Two parameter % Tail DNA and Olive tail moment were used to analyse data by kinetic imaging software (Andor Technology Ltd, Belfast, UK).

2.13 Polymerase Chain Reaction (PCR)

The principle of the PCR technique depends on repeated cycles of DNA sequencing under various temperatures in three different steps. One DNA fragment is used to yield two copies, then four, then eight, and so on. Three major steps that rely on changes in temperature are documented in one PCR cycle. In the first step, the double strand of DNA is denatured and separated at 90–97°C. Strengthening occurs

at 55–65°C, which is the second step. The extension takes place at 72°C, which is the third step. The PCR experiment needs the following reagents: a specific protein called polymerase (Taq polymerase); a DNA template (i.e., the target protein); a small DNA fragment, also called the primer, to attach the building blocks; nucleotides (i.e., guanine, cytosine adenine, and thymine), and a reaction buffer. If these components are accessible, the enzyme produces typical copies of the template (Joshi and Deshpande, 2011).

Isolated lymphocytes from fresh blood were used in both PCR and Western blots techniques. Blood was diluted with (0.9% NaCl) 1:1, with gentle mixing using 50ml graduated tube. 6 ml from the mixture was placed in a 15ml Falcon tube containing 3ml of Lymphoprep™ all the tubes were centrifuged 20 minutes at 600xg. Once the centrifugation was completed the mixture was divided into three layers, red layer at the bottom then, Lymphoprep, white layer which is isolated lymphocytes and plasma layer at the top. The white layer was collected and transferred to another Falcon™ tube and washed with saline. The washed cells were centrifuged at 375g for 15 minutes. White pellets were suspended, and cultured lymphocytes were cultured at a concentration of 200,000 cells in 1 ml of medium RPMI 1640 glutamax® containing 25mm hepes, 75ml foetal bovine serum for 24 hours. 10 µl of PhIP 140µM final concentration was used to induce DNA damage of cultured lymphocytes for two hours.

2.13.1 RNA Extraction

Cultured lymphocytes ($2x10^5$cells/ml) were mixed with (EL) buffer using Falcon™ tube to obtained RNA from cultured lymphocytes. The isolated RNA was stored at -80°C for further use.

2.13.2 Synthesis cDNA

The iScript™ cDNA Synthesis Kit was used to prepare the cDNA from lymphocyte RNA. First step: 10 µl of extracted RNA was mixed with 4 µl 5XisCript reaction buffer plus 1 µl of Improm II RT (reverse transcriptase) and 5 µl nuclease-free water. All the components were mixed together in small Eppendorf® tubes in ice. Second step: all the Eppendorf® tubes were transferred and incubated using a Thermocycler machine for the first incubation: Priming 5 min at 25°C. Then the reverse transcriptase step for 20min at 46°C. Subsequently, RT was activated for 1 minute at 95°C. Lastly the Eppendorf® tubes were holed in a PCR thermo cycler at 4°C for 10 minutes. Once the machine stopped the prepared cDNA was storaged at -20°C for further use.

2.13.3 Quantitative real time PCR analysis (QPCR)

The procedures of qPCR included preparation of mixture 1 which has a primary end volume of 12 µl; this amount was enough for 24 wells. Mixture 2 which is β actin. Mixture 3 which is cDNA as prepared before; all mixtures were mixed in separate Eppendorf® tubes. The experiments were designed in triplicate with both primary and Fast SYBR® Green for the confirmation of the results. PCR max ECOPLATE 48 was used to mix all the mixtures 1, 2 and 3 and covered by Eco 48 real time PCR

seals. Mixtures were immediately vortexed and a plate spinner to collect the mixtures at the bottom of the plate and air bubbles were removed. The plate was put into an ECO 48 Real-Time PCR machine; also, the proposed protocol was followed. All experiments were done in triplicate wells. For each experiment, β actin was utilized as a housekeeping gene. ΔCq value was calculated. The gene expression was normalised against β actin; the ΔΔCq was further calculated for gene expression and normalised against untreated cells to evaluate the gene expression fold. The data of qPCR such as ∆Cq and ΔΔCq values were analysed using SPSS and Graph bad prism 6 to evaluate gene expression. The design was repeated with other primaries such as COX1, COX2 and Bcl-2. The ΔCq and ΔΔCq was calculated according to the method in ECO 48 qPCR.

2.14 Western blot Technique

2.14.1 Cell culture and protein isolation

Once lymphocytes were isolated from whole blood of TB patients or healthy individuals, the cells were cultured in 6 well cell culture plates for 24 hours. Each well contained 1 ml of prepared GlutaMAX RPMI 6140 and 26 µl of PHA for 200,000cells/well. After 24 hours the DNA damage was induced in lymphocytes by addition of 10 µl of PhIP (140µM final concentration) for half hour. The lymphocytes were then treated with10 µl of 100 µM of quercetin bulk and nano forms for two hours. After the treatment the medium was removed using a pipette and the wells were washed twice with 2 ml of lysis buffer to lyse the cells and scrub de-attach the cells using a plastic scrubber. All suspensions from each well were transferred to Eppendorf tubes. The cell suspension was heated for 10 min at 90°C in a water bath

and stored in the - 20 °C freezer. The isolated protein measurement was performed using the Bradford protein assay (Bradford, 1976; Ernst and Zor, 2010).

2.14.2 Isolated protein calculation

The Bio-Rad Bradford assay kit was used to measure isolated protein form each sample according to the manufacture's guidelines. The following table illustrates the protein assay experimental design.

Reagent	**Blank (Reagent only)**	**Standardconcentration 50 mg**	**Untreated cells**	**PhIP 140µM**	**10 µl PhIP 140µM + 10µl of 100 µM Bulk treatment**	**10 µl PhIP 140µM+ 10µl of 100 µM nano treatment**
Bradford assay reagent 1	250 µl	250 µl+0.625 µl from stander	250 µl	250 µl	250 µl	250 µl
	250 µl	250 µl+1.25 µl+ standard				
	250 µl	250 µl+ 2.5 µl standard				
	250 µl	250 µl+5 µl standard				
	250 µl	250 µl+10 µl standard				
Sample	----------	----------	5 µl Isolated protein	5µl Isolated protein	5 µl Isolated protein	5 µl Isolated protein

Table7: illustrates Bio-Rad Bradford protein assay.

The reagent and sample were mixed and incubated for 5 minutes in the dark room. The MRX-Microplate reader was used to measure protein concentration in each sample. Each sample was evaluated in triplicate. The absorbance calculated

spectrophotometrically at 540 nm using a microplate reader. Protein concentrations were measured depending on standard concentrations.

2.14.3 Gel preparation and electrophoresis

Glasses were washed with water, dried, and then fixed in cassettes**.** Each cassette with glasses was placed on the Perspex rack holder. 5 ml of spreading gel (6.67 ml 30% acrylamide, 5 ml ddH2O, 4 ml 1.5 M Tris pH 8.8, 160 µl 10% APS, 160 µl 10% SDS, and 16 µl TEMED) was injected between glasses using 1 ml pipette until it reached the green edge of cassette. The spreading gel and glasses were kept at room temperature for one hour to solidify, then 1 ml from stacking gel (freshly prepared) (2 ml 30% acrylamide, 5.3 ml ddH2O, 2.5 ml 1.5 M Tris pH 8.8, 100 µl 10% APS, 100 µl 10% SDS, and 10 µl TEMED) was injected using 1 ml pipettes to the top of each cassette. A comb was inserted between glasses and kept at room temperature for 45 minutes until complete gelation. Cassettes and glasses were adjusted on the electrophoresis plate holding rack which fixed in the electrophoresis tank. Freshly prepared running buffer (30.29 g Tris base 25 mM, 142.63 g glycine190 mM and 10.0 g SDS 0.1% in 1L distilled water) was added in the electrophoresis tank, combs were removed. Samples were mixed with laemmli buffer (10% 2-mercaptoethanol, 4% SDS, 20% glycerol, 0.004% bromophenol blue and 0.125 M Tris-HC) at a rate dependent on the concentration of total protein in each sample. 30 µl of each prepared sample was loaded into one of the wells. At 200 V 300 AM the electrophoresis was run for one hour.

2.14.4 Transferring the protein from the gel to the Polyvinylidene difluoride (PVDF) membrane

The membrane was cut into a small size that covered the gel size, dipped in methanol 70% for 30 seconds and then transferred to distilled water for 1 minute. Transferring buffer was freshly prepared (14.26 g glycine 190 mM, 3.02 g Tris base 25 mM, 200 ml methanol in 1L distilled water). The components were mixed together in a clean and dry beaker, 3000 ml capacity placed on a magnetic stirrer at room temperature. The gel holding the electrophoresed protein and PVDF membrane were attached together and covered by a filter paper on both sides and finally protected by two pieces of a sponge on both sides. The air bubbles in the sandwich were removed by pressing one side of sponge using a 1ml pipette. The membrane, gel, filter paper, sponges were assembled in the transfer sandwich. The sandwiches were fixed to blot connect to the cathode, and the gel was linked to the anode. The cassette was fixed in the tank containing transfer buffer and ice block, then the power supply was adjusted at 100 V, 300 AM and run for 1 hour.

2.14.5 Antibody staining

Once the transferring time was completed the PVDF membrane was moved to sterilised square petri dish containing blocking buffer (KCl 50mg +NaCl 2g + Trisma Base 57mg were dissolved in 250 ml dH_2O, pH 7.4 then 250 µl of Tween 20 and PSA 9g 4% were added). The square petri dish was fixed on a rotator in a cold room for 1 hour. Then the membrane was transferred to another square petri dish containing 7.5 µl of primary antibody diluted with 15 ml of blocking buffer and incubated overnight on a rotator in the dark cold room. Subsequently, the membrane was washed by washing solution consisted of (PBS/0.05%-0.1% Tween-20) three

times for 5 min each. After that, the membrane was transferred to Squair Petri dish contained secondary antibody diluted by Blocking buffer 1:2000 and incubated in dark place for one hour at room temperature under rotating.

2.14.6 Bands imaging

After the secondary antibody incubation time finished, the membranes were washed three times five minutes each time by washing solution. 5ml from reagent 1and reagent 2 (ECL) were prepared in the dark room. The membrane was covered by prepared (ECL) in dry and clean square petri dish and placed on the rotator for five minutes. An Image was captured by darkroom development techniques for chemiluminescence or normal image scanning methods for colorimetric examination.

2.14.7 The re-staining of membrane

The membrane was immersed for ten min in 35 ml of re-staining solution, after that the volume was completed up to 1litre. The membrane was then transferred to the square petri dish containing PBS for ten minutes, this step was repeated twice. Subsequently, the membrane was placed in 35ml of TSBT solution. The entire components were dissolved in 250 ml dH2O and the pH fixed at 7.4, then 250 µl of Tween 20 and PSA 9g 4% were added for 5 min, this step was repeated twice. Then follow the same procedures.

2.15 Statistical analysis

Twenty blood samples and twenty sperm samples from healthy individuals were used in the Comet assay for control samples. Three fresh blood samples from healthy individuals and three fresh samples from TB patients were used in the

CBMN assay. Ten blood samples were used from each group of TB patients and healthy individuals to investigate genotoxicity of food the mutagens (PhIP) and (IQ). Three fresh blood samples from healthy individuals and TB patients were cultured and were used to evaluate the catalase protein by the Western blot technique and COX1, COX2, P53 and Bcl-2 using qPCR. Each sample for Western blot and qPCR were repeated in triplicate to confirm the results. The means of group data were calculated with standard errors. For qPCR, the ΔCq and ΔΔCq method were used, which is a suitable way to study the relative changes in gene expression from real-time quantitative PCR experiments. The normal distribution of the data was examined by the Kolmogorov-Smirnov and Shapiro Wilk's test to select the correct parametric. A test One Way ANOVA test was used to compare untreated cells and TB blood samples in the Comet assay, Western blot, and qPCR, while the T-test was used in the CBMN assay.

The variances between groups were examined by the Kruskal-Wallis method and Mann-Whitney U-test. Concentration response correlations were detected using Pearson's test for both nano and bulk forms of quercetin. The value of $p \leq 0.05$ was selected as statistically significant. All analyses were created by Graph pad prism 6 and SPSS for Windows statistical package (version 18.0).

3 Chapter (3) The Comet assay in sperm and peripheral lymphocytes after treatment with quercetin bulk and nano form, results, graphs and statistical analysis

3.1 Introduction

Many studies have examined the influence of antioxidant as a protective compound in respiratory diseases (Nguyen et al., 2004; Habas et al., 2018). Bischoff (2008) reported that the regular intake of a quercetin dose could help to prevent and protect certain illnesses. Kandaswami and Middleton Jr. (1994) reported that flavonoids characteristics could protect cells from lipid peroxidation and oxygen free radicals. Also, complex pathological conditions such as cancer can arise due to oxygen free radical and lipid peroxidation. Accordingly, antioxidants could contribute to preventing cancer and chronic inflammation (Haliwell, 1994). The average dietary intake of flavonoid was calculated by per cent, where tea is a principle source occupying 48% of total intake, while onions take 29%, but apples only have 7%. The lowest amount was with fruit and vegetables (Hollman and Katan, 1999). There are conflicting reports on the daily dietary requirements of flavonoids. For example, Hollman and Katan in 1999 described that the human body needs a small amount daily intake of flavonoids which is a few hundred milligrams. Kühnau in 1976 reported that 650 mg/day from flavonoid were required.

Many of studies examined the action of antioxidants where levels of oxidative stress are reduced by inequality of the two anions hydrogen peroxide and (superoxide) from oxygen, while quinones and semiquinones can also damage DNA directly (Bolton, 2002). Therefore, producing high levels of free radicals can induce DNA damage in a pro-oxidative state by producing chromosomal instability (Gibellini et al., 2010). However, quercetin has a strong ability to scavenge free radicals by suppression of lipid peroxidation (Bentz, 2009). In the last twenty years, a noticeable improvement has been presented in methods which can identify and estimate DNA

damage. Wu and Jones in 2012 pointed out the single strand DNA breaks in sperm and peripheral lymphocytes were assessed by the Comet assay. In the present study sperm and peripheral lymphocytes that were collected from healthy individuals and lymphocytes obtained from TB patients were embedded in to slides pre-coated by agarose gels 5% and positioned horizontally in an electrophoresis tank which had alkaline conditions at pH 13. During electrophoresis, fragments of DNA travelled to the anode (+ve) because they have a negative charge and migrated faster compared undamaged DNA. This method created a comet shape which was stained with ethidium bromide and examined under a fluorescence microscope. DNA damage was measured in terms of the % Tail DNA and Olive tail moment (Wu and Jones, 2012).

Lymphocytes were collected from healthy individuals and examined using the MTT assay and only samples with over 80% viable cells were used in the experiments. The lymphocytes from the healthy individuals were then treated with 60μM hydrogen peroxide in the presence of three different concentrations of bulk form of quercetin (10μM, 25μM, and 100μM) and also similar concentrations of the nano form of quercetin. The positive control Eppendorf® contained 60μM of H_2O_2 in lymphocytes, because this concentration produced the highest damage in DNA without causing toxicity, while in sperm 50 μM of H_2O_2 was selected as a positive control. The lower concentration of the positive control in the sperm could be because of the higher sensitivity of sperm to H_2O_2 and contained half the number of chromosomes. Lymphocytes collected from TB patients were incubated with 60μM of H_2O_2 and the same concentrations of bulk and nano forms of quercetin as in the case of lymphocytes in healthy individuals. Once the individuals were examined, data were

analysed by special software (cuhat software) to estimate the influence of both forms on reducing DNA damage.

3.2 Materials and Methods

All the materials and methods used in this chapter have been shown and described in chapter 2, section 2.11.1 to 2.11.5-page number 53-54.

3.3 Scoring and result analysis

All slides were scored by a fluorescence microscope connected to the computer and camera designed with Comet 6 software (Andor Technology Ltd, Belfast, UK). All the procedures related to this assay have been explained in chapter 2 section 2.15-page number 70. Graph pad prism 6 and SPSS for Windows statistical package (version 18.0) were used to calculate the P value (≤0.05) of % Tail DNA and Olive tail moment to determine the level of significance.

3.4 Results

3.4.1 Results of tetrazolium dye (MTT) assay

The lymphocytes collected from three different healthy individuals were treated with quercetin bulk and nano forms at different concentrations 10 µM, 25 µM, 100 µM to investigate cytotoxicity of quercetin using the MTT assay. The average of three experiments from three different healthy individuals was calculated and represented by a line graph to show cell survival. It was clear that the percentage of cells survival obtained from quercetin treatments were rated from 95.8% to 97.9%. The data were calculated based on negative control absorbance as described in chapter 2 section 2.8-page number 51. The results confirm that the quercetin bulk and nano form concentrations were safe to use in this study.

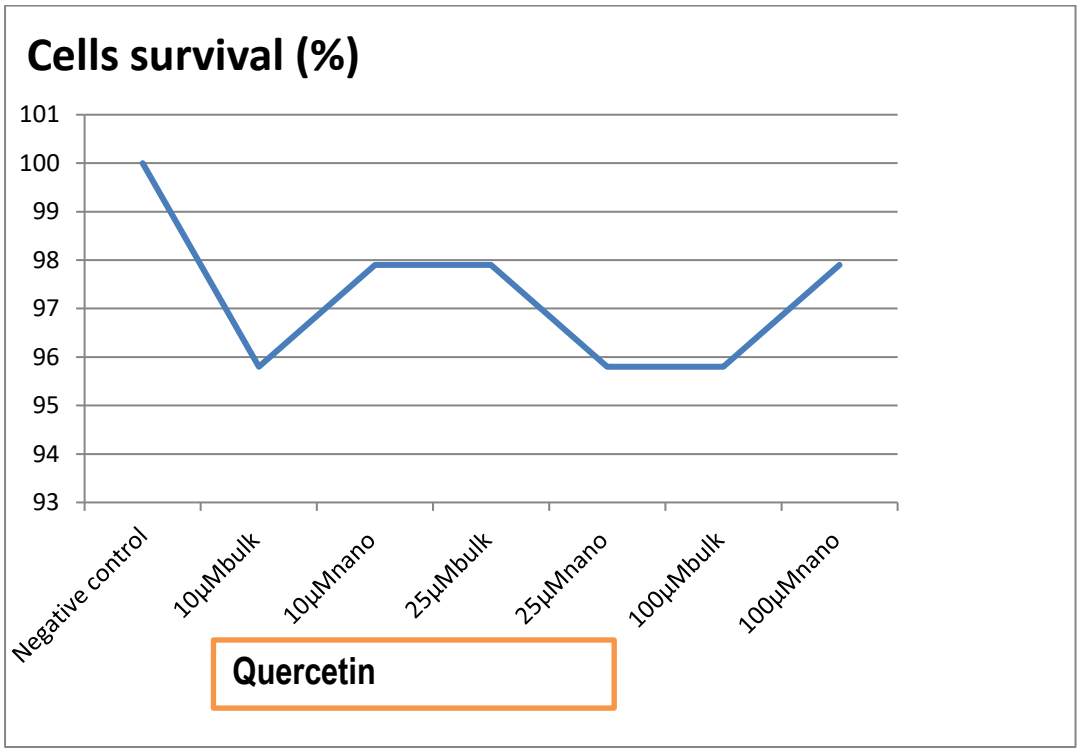


Figure 13: Graph showing the means of cell survival of lymphocytes collected from three different healthy individuals treated with quercetin at different concentrations (donors=3).

3.4.2 Comet assay results

3.4.3 Control samples sperm and lymphocytes

The Comet assay was performed on 20 sperm and 20 blood samples collected from different healthy individuals. All the sperm samples were processed as per the guidelines in the WHO laboratory manual for the examination and processing of human semen and blood samples, they were diluted 1:1 with RPMI1640 medium; and all the samples were stored at -80°C. Twenty dose response experiments each sample was performed for sperm and peripheral blood lymphocytes after treatment with different concentration of hydrogen peroxide to determine positive controls in this study. All the experiments have been assessed by Comet assay to detect DNA damage based on %Tail DNA and Olive tail moment. The Average of % Tail DNA and Olive tail moment of the 20 healthy donors were calculated for sperm and lymphocytes that were treated by hydrogen peroxide and the concentration responses were determined. All the concentrations were compared to the negative control value.

3.4.4 Dose response experiments with lymphocytes form healthy individuals

The DNA damage reached the peak at 65μM of H_2O_2 Figures 14 and 15. The striking features are that 65μM, and 55μM of H_2O_2 showed the maximum damage for both parameters % Tail DNA and Olive tail moment. Furthermore, the Graph pad one-way ANOVA test showing high significance ***$P \leq 0.001$ for these concentrations of H_2O_2. This prompted the use the mean value i.e.60μM of H_2O_2 as the concentration for positive control for experiments involving lymphocytes.

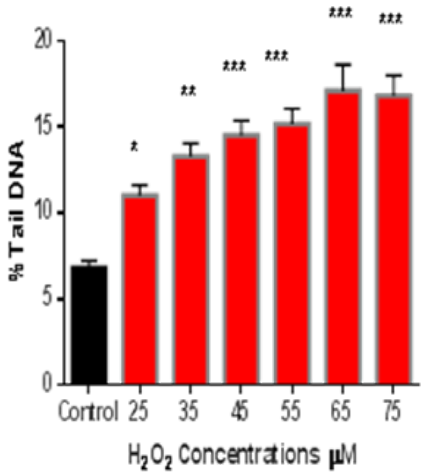


Figure 14 : Different concentrations of H_2O_2 presented as means of % Tail DNA treated lymphocyte collected from 20 different healthy individuals, ± SEM One-Way AVOVA test and significance compared with the untreated cells (donors=20).

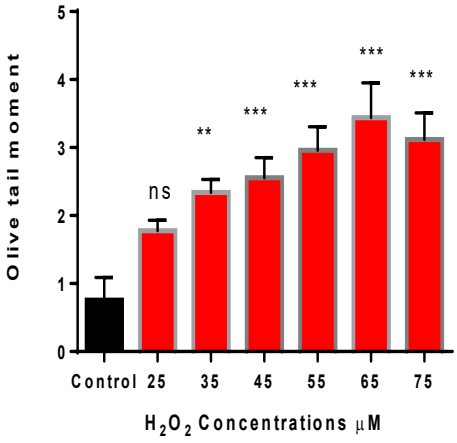


Figure 15: Different concentrations of H_2O_2 presented as means of Olive tail moment treated lymphocytes collected from 20 different healthy individuals, ± SEM, One-Way AVOVA test and significance compared with theuntreatedcells(donors=20).

3.4.5 Dose response experiments with sperm from healthy individuals

In the case of sperm, the DNA damage reached the peak at concentrations of, 45µM and 55 µM of H_2O_2, and generated highly significant DNA damage (figures 16 and 17). This confirmed that the statically significant calculation using Graph pad prism One-Way ANOVA test gave a P value ***P ≤ 0.001 compared to the negative control. Consequently, the mean of two concentrations is 50 µM of H_2O_2 that why taken as a positive control of sperm experiments without toxicity (Henderson, Albertini and Aardema, 2000).

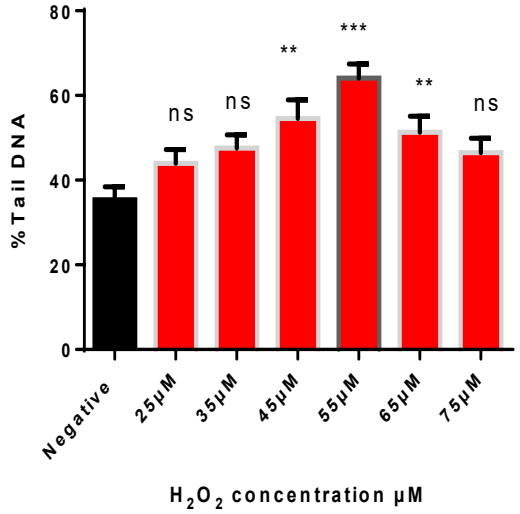


Figure 16: Different concentrations of H_2O_2 presented as means of % Tail DNA treated sperm collected from 20 different healthy individuals, ±SEM One-Way AVOVA test and significance compared with the untreated cells (donors=20).

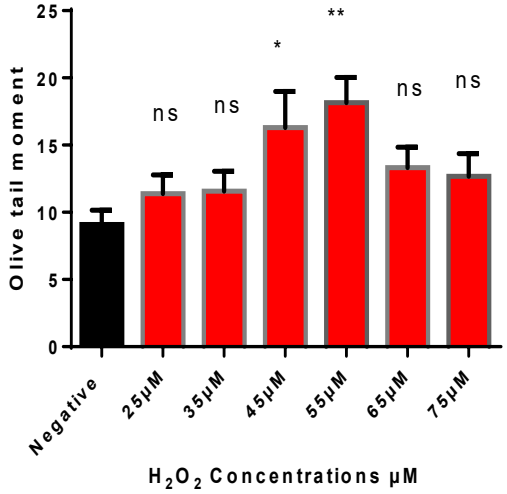


Figure 17: Different concentrations of H_2O_2 presented as means of Olive tail moment treated sperm collected from 20 different healthy individuals, ±SEM One-Way AVOVA test and significance compared with the untreated cells (donors=20).

3.4.6 Effect of quercetin bulk form on DNA damage induced by H_2O_2 in lymphocytes collected from healthy individuals.

This experiment involved the use of lymphocytes from 10 healthy individuals. Figures 18 and 19 illustrate how different concentrations of the bulk form of quercetin reduce DNA damage induced by H_2O_2 in lymphocytes collected from healthy individuals. It is evident that the influence of quercetin bulk was not significant statistically using both % tail DNA and Olive tail moment as the P value was ≥0.05 compared to the positive control. The figures include the mean value of ten individuals showing the standard error and significance using a One-Way ANOVA test. All concentrations of quercetin and controls were normally distributed.

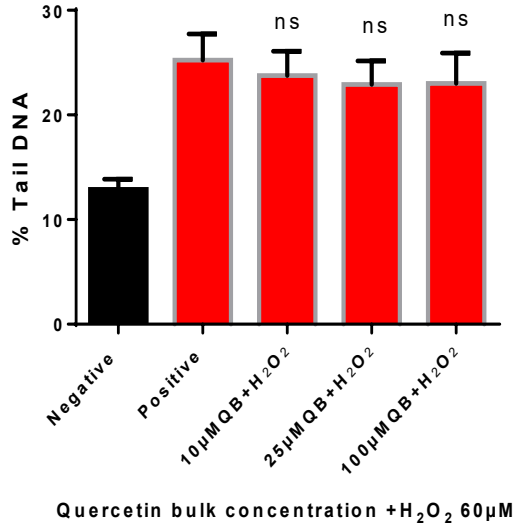


Figure 18: The effect of quercetin bulk on DNA damage presented as mean of % Tail DNA of lymphocytes collected from 10 different healthy individuals treated with quercetin bulk in the presence of H_2O_2, (60µM), ± SEM One-Way AVOVA test and significance compared with the positive control (donors=10).

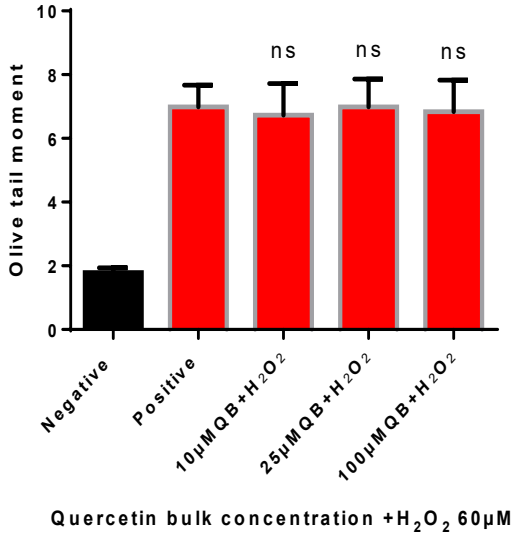


Figure 19 : The effect of quercetin bulk on DNA damage presented as mean of Olive tail moment of lymphocytes collected from 10 different healthy donors treated with quercetin bulk in the presence of H_2O_2 (60µM), ± SEM One-Way AVOVA test and significance compared with the positive control (donors=10).

3.4.7 Effect of quercetin nano form on DNA damage induced by H_2O_2 in lymphocytes collected from healthy individuals

This experiment involved the use of lymphocytes from 10 healthy individuals. Figures 20 and 21 depict the influence of quercetin nano on DNA damage that was caused by hydrogen peroxide in lymphocytes obtained from healthy individuals. Overall perspective, in both figures 15 and 16 all the concentrations of quercetin nano were not significant using % tail DNA and Olive tail moment based on one-way ANOVA test. The comparison between each concentration and the positive control display P value ≥0.05. Even the concentration of 100µM was not significant. The negative control, positive control (60µM H_2O_2) and quercetin concentrations were normally distributed.

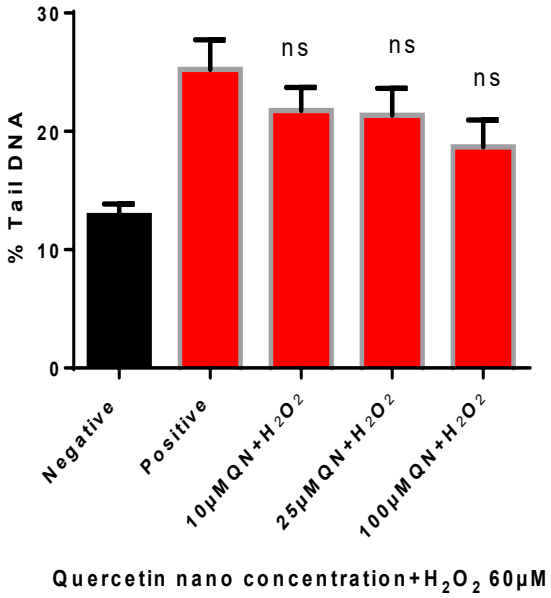


Figure 20 : The effect of quercetin nano on DNA damage presented as means of % Tail DNA of lymphocytes collected from 10 different healthy individuals treated with quercetin nano in the presence of H_2O_2 (60µM) ± SEM One-Way AVOVA test and significance compared with the positive control (donors=10).

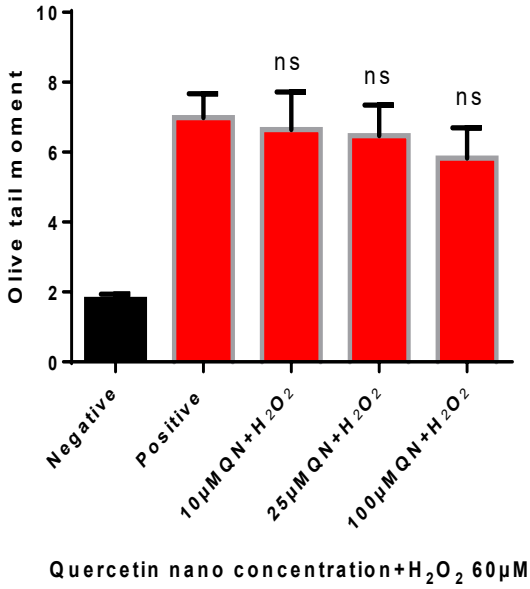


Figure 21 : The effect of quercetin nano on DNA damage presented as means of Olive tail moment of lymphocytes collected from 10 different healthy individuals treated with quercetin nano in the presence of H_2O_2 (60µM) ±SEM One-Way AVOVA test and significance compared with the positive control (donors=10).

3.4.8 Effect of quercetin bulk on DNA damage induced H_2O_2 in lymphocytes from patients with TB

Both figures 22 and 23 provide information on the effect of different concentrations of quercetin bulk on the lymphocytes from a patient with TB. All the concentrations reduce DNA damage caused by H_2O_2, however; the reductions were non-significant as the P value was ($P \geq 0.05$) based on a one-way ANOVA test in both the parameters namely the % tail DNA and Olive tail moment.

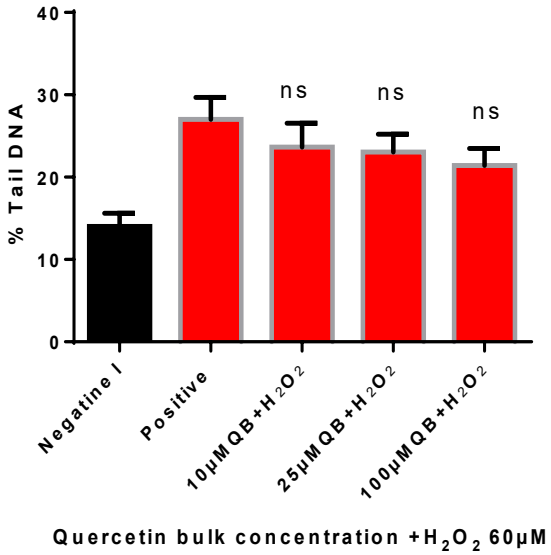


Figure 22 : The effect of quercetin bulk on DNA damage presented as means of % Tail DNA of lymphocytes collected from 10 patients with TB treated with quercetin bulk in the presence of H_2O_2 (60µM), ± SEM One-Way AVOVA test and significance compared with the positive control (donors=10).

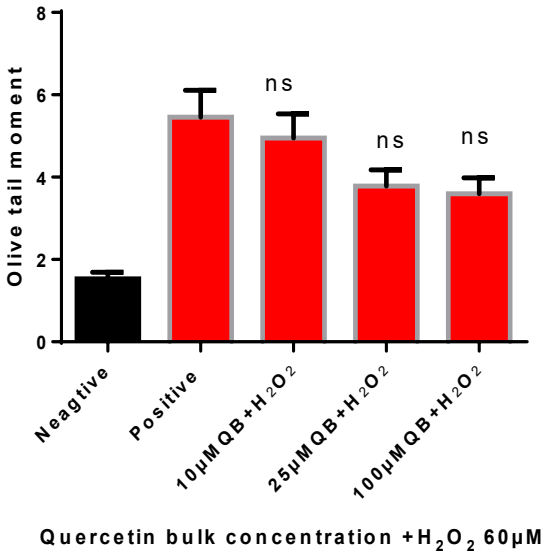


Figure 23: The effect of quercetin bulk on DNA damage presented as means of Olive tail moment of lymphocytes collected from 10 patients with TB treated with quercetin bulk in the presence H_2O_2 (60µM), ± SEM One-Way AVOVA test and significance compared with the positive control (donors=10).

3.4.9 Effect of quercetin nano on DNA damage induced H_2O_2 in lymphocytes from patients with TB.

Figures 24, 25 illustrate information about lymphocytes which are collected from patients with TB induced DNA damage by 60µM of H_2O_2 and treated with quercetin nano. It was obvious that from both bars graph the DNA protection was more significant at 100µM of quercetin nano compared to the positive control, whereas the concentration 10µM was not significant in both parameters of % tail DNA and Olive tail moment. The best reduction of DNA damage was at the same concentration in both parameters. All the concentrations were compared to the positive control using the one-way ANOVA test. Furthermore, statistically, the concentration 100µM of quercetin nano has a P value *** $P \leq 0.001$.

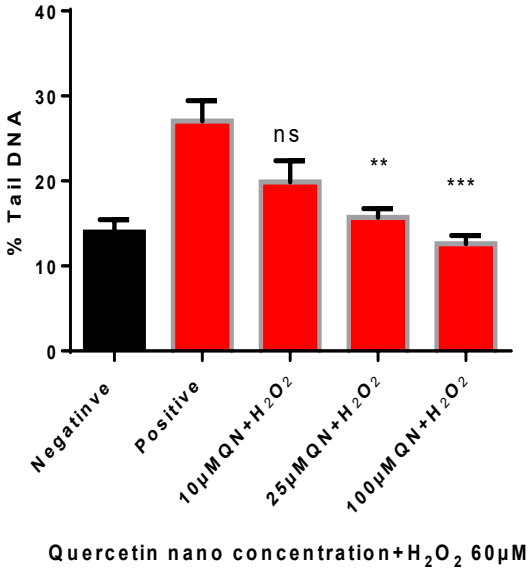


Figure 24: The effect of quercetin nano **on** DNA damage presented as mean of % Tail DNA of lymphocyte collected from 10 TB patients treated with quercetin nano in the presence of H_2O_2 (60µM), ± SEM One-Way AVOVA test and significance compared with the positive control (donors=10).

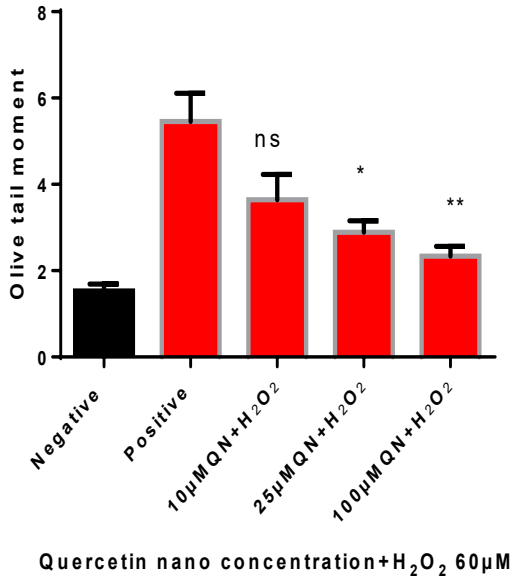


Figure 25: The effect of quercetin nano **on** DNA damage presented as mean of Olive tail moment of lymphocytes collected from 10 TB patients treated with quercetin nano in the presence of H_2O_2 (60µM), ± SEM One-Way AVOVA test and significance compared with the positive control(donors=10).

3.4.10 Effect of quercetin bulk in reducing DNA damage induced by H_2O_2 in sperm collected from healthy individuals.

Figures 26 and 27 illustrate data of healthy sperm treated by quercetin bulk in the presence 50µM of H_2O_2. All concentrations of quercetin in reduced DNA damage were not significant statistically in DNA protection when compared with the positive control in both parameters, % tail DNA and Olive tail moment the P value was P ≥0.05 based on one-way ANOVA test. All concentrations and both controls were distributed normally.

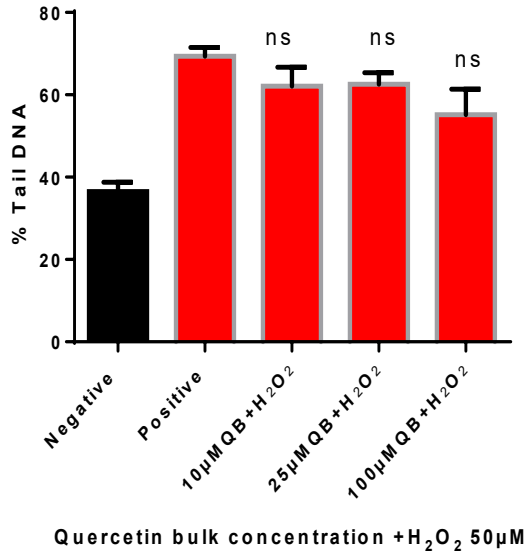


Figure 26: The effect of quercetin bulk on DNA damage presented as mean of % tail DNA of sperm collected from 10 healthy individuals treated with quercetin bulk in the presence of H_2O_2 (50µM) ± SEM One-Way AVOVA test and significance compared with the positive control (donors=10).

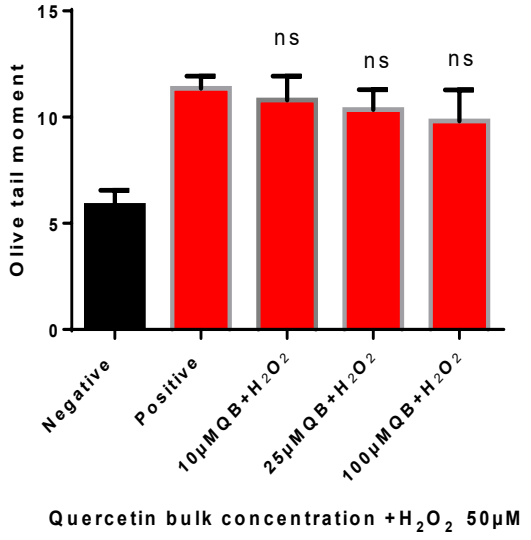


Figure 27: The effect of quercetin bulk on DNA damage presented as mean of Olive tail moment of sperm collected from 10 healthy individuals treated with quercetin bulk in the presence of H_2O_2 (50µM) ± SEM One-Way AVOVA test and significance compared with the positive control (donors=10).

3.4.11 Effect of quercetin nano in reducing DNA damage induced by H_2O_2 in sperm collected from healthy individuals

Figures 28 and 29 illustrate information about the effect of the nano form of quercetin on sperm collected from healthy individuals in the presence of 50µM of H_2O_2. It was clear that there was significant protection of DNA damage by quercetin nano. The protection was observed at concentrations ranging from 25µM to 100µM respectively using % tail DNA, whereas the concentration 10µM was non-significant in both parameters Olive tail moment and % tail DNA. The two parameters display the maximum reduction in DNA damage at a concentration of 100µM of quercetin nano. The statistical analysis using the one-way ANOVA test showed the P value was ****P* 0.001 for the highest concentration.

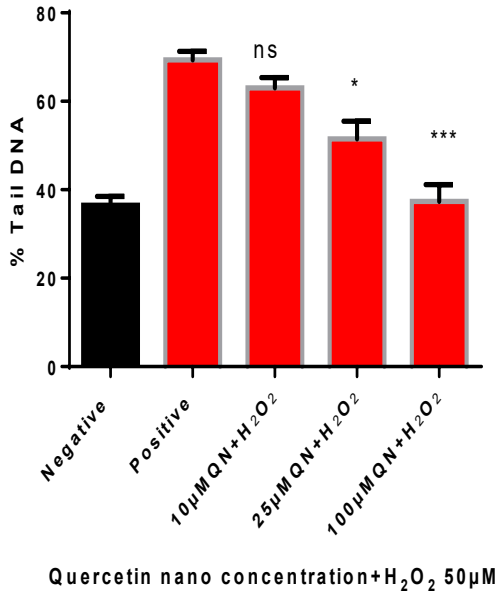


Figure 28 : The effect of quercetin nano on DNA damage presented as mean of % Tail DNA of sperm collected from healthy individuals in the presence of H_2O_2 (50µM), ± SEM One-Way AVOVA test and significance compared with the positive control (donors=10).

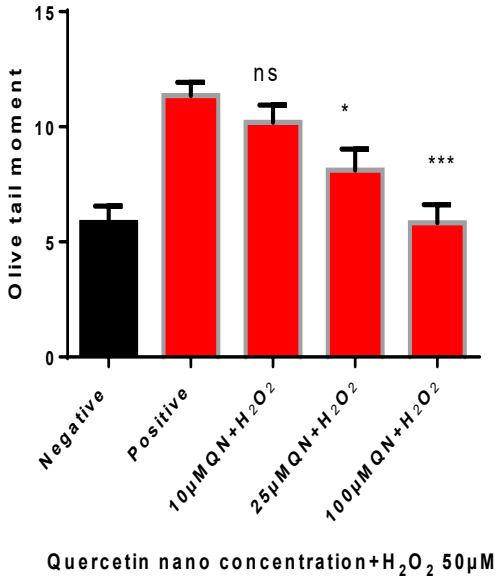


Figure 29: The effect of quercetin nano on DNA damage presented as mean of Olive tail moment treated sperm collected from healthy individuals in the presence of (50µM), H_2O_2 ± SEM One-Way AVOVA test and significance compared with the positive control (donors=10).

3.4.12 Comparison of the DNA protective effect of quercetin bulk on DNA damage induced by H_2O_2 in lymphocytes and sperm collected from healthy individuals.

Figures 30 and 31 illustrate a comparison between sperm (figures 26and 27) and lymphocytes (figures 18 and 19) from healthy individuals treated with quercetin bulk in the presence of H_2O_2. The DNA damage was analysed using two parameters, the % tail DNA and Olive tail moment. Using the two-way ANOVA test, the reduction was non-significant in both sperm and lymphocytes collected from healthy individuals with all concentrations in both parameters with the P values≥ 0.05.

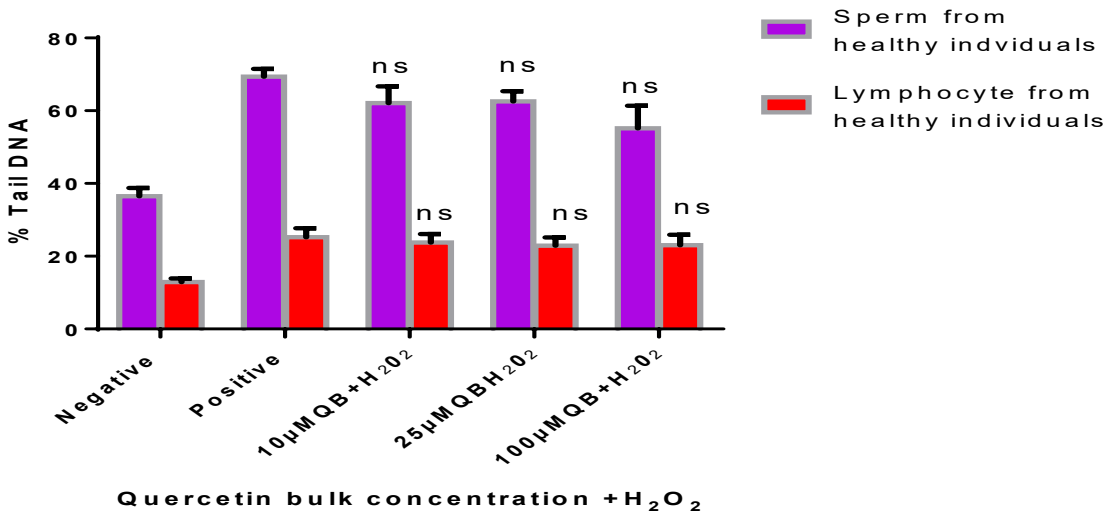


Figure 30 : A comparison between % Tail DNA of sperm (figure 26) treated with 50µM H_2O_2 and lymphocytes (figure 18) treated with 60 µM H_2O_2 collected from 10 different healthy individuals treated with quercetin bulk in the presence of H_2O_2, ± SEM, Two-Way AVOVA test and significance compared with the positive control (donors=10).

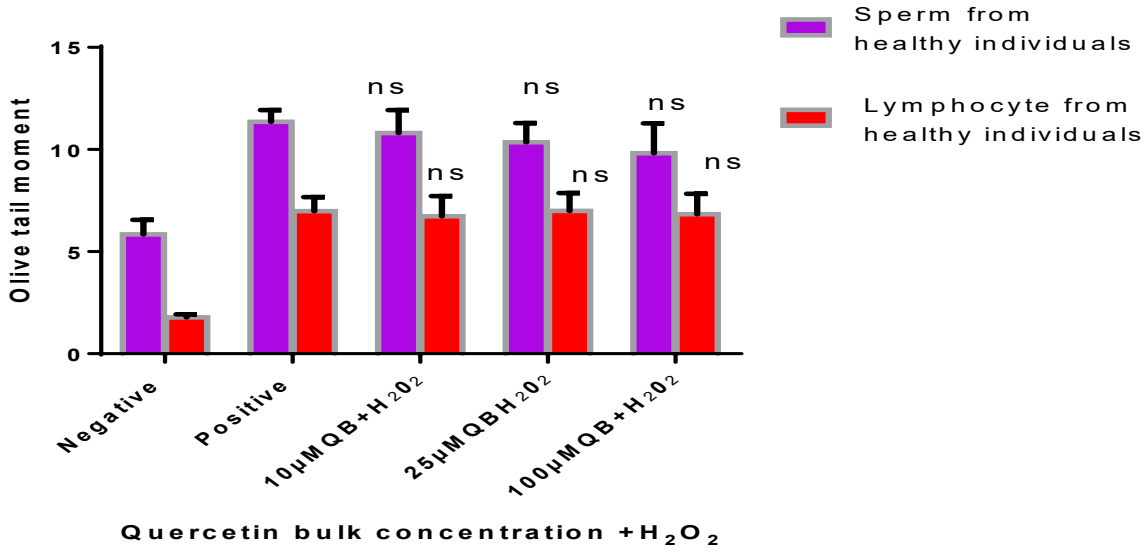


Figure 31: A Comparison between Olive tail moment of sperm (figure 27) treated with 50µM H_2O_2 and lymphocytes (figure 19) treated with 60 µM H_2O_2 collected from 10 different healthy individuals treated with quercetin bulk in the presence of H_2O_2, ± SEM Two-Way AVOVA test and significance compared with the positive control (donors=10).

3.4.13 Comparison of the DNA protective effect of quercetin nano on DNA damage induced by H_2O_2 in lymphocytes and sperm collected from healthy individuals

Figures 32 and 33 depict a comparison of the DNA protective effect of three concentrations of quercetin nano between lymphocytes (figure 20 and 21) and sperm (figure 28 and 29) collected from healthy individuals in the presence of H_2O_2. The influence of quercetin was examined in both diploid and haploid cells to evaluate DNA protection by nano form. In sperm, there was the gradual reduction of DNA damage because of quercetin nano beginning from a concentration 25µM to a concentration of 100µM where the P value was ***$P \leq 0.001$ for both the parameters. In contrast in the lymphocytes collected from healthy individuals, both % tail DNA and Olive tail moment quercetin effect on DNA damage which was not significant at all concentrations which registered P values ≥0.05 in a two-way ANOVA test. All concentrations and both controls were distributed normally.

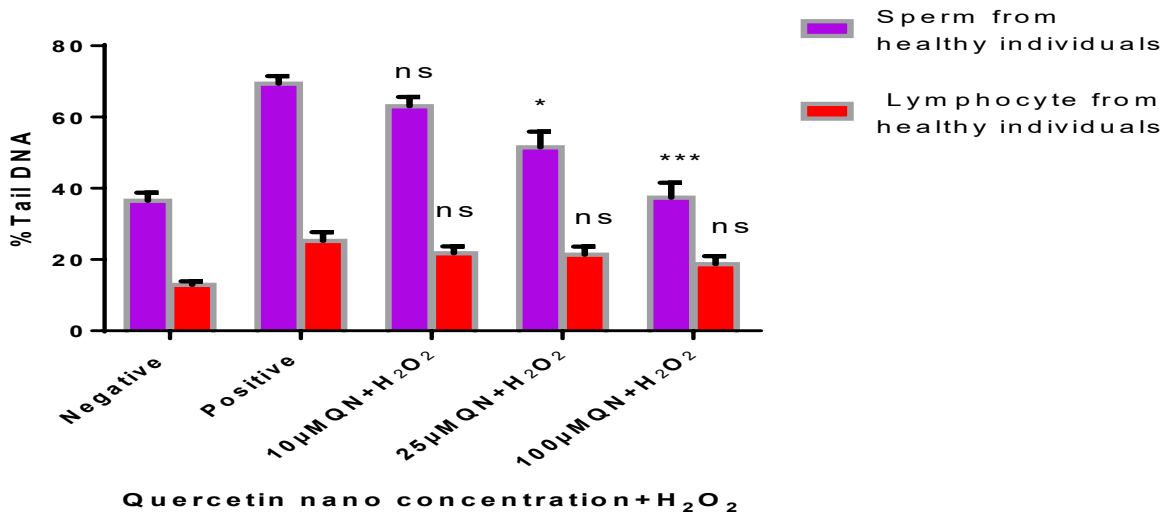


Figure 32 : A Comparison of % Tail DNA between sperm (figure 28) treated with 50µM H_2O_2 and lymphocytes (figure 20) treated with 60 µM H_2O_2 collected from 10 different healthy individuals treated with quercetin nano in the presence of H_2O_2, ± SEM Two-Way AVOVA test and significance compared with the positive control (donors=10).

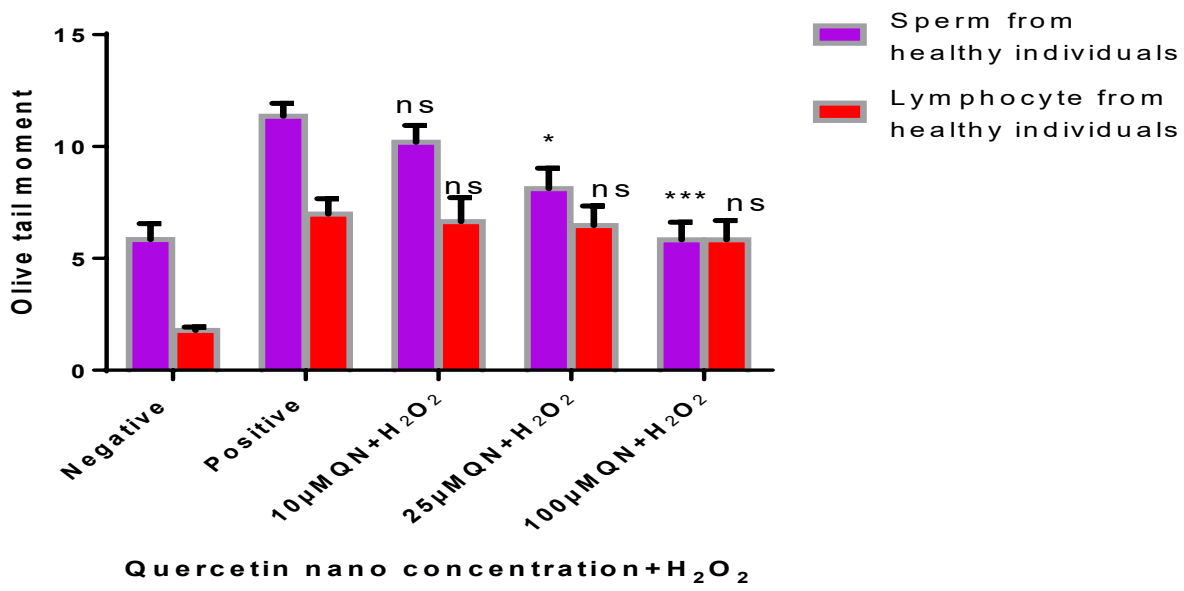


Figure 33: A comparison of Olive tail moment between sperm (figure 29) treated with 50µM H_2O_2 and lymphocytes (figure 21) treated with 60 µM H_2O_2 collected from 10 different healthy individuals treated with quercetin nano in the presence of H_2O_2, ± SEM Two-Way AVOVA test and significance compared with the positive control (donors=10).

3.4.14 Comparison of the DNA protective effect of quercetin bulk on DNA damage induced by H_2O_2 in lymphocytes collected from healthy individuals and patients with TB.

Figures 34 and 35 show a comparison between lymphocytes collected from healthy individuals (figure 18 and 19) and lymphocytes from patients with TB (figure 22 and 23). The DNA damage was described by two parameters % tail DNA and Olive tail moment. The DNA damage reduction was compared to the positive control; in both the groups, lymphocytes were treated with three concentrations of quercetin bulk. The % tail DNA (figure 29) shows a non- significant effect of three concentrations of quercetin bulk in lymphocytes from healthy individuals as well as lymphocytes collected from TB patients. The concentration 100µM of quercetin bulk shows non-significant reduction in the DNA damage. However, this reduction was statistically non-significant in both parameters

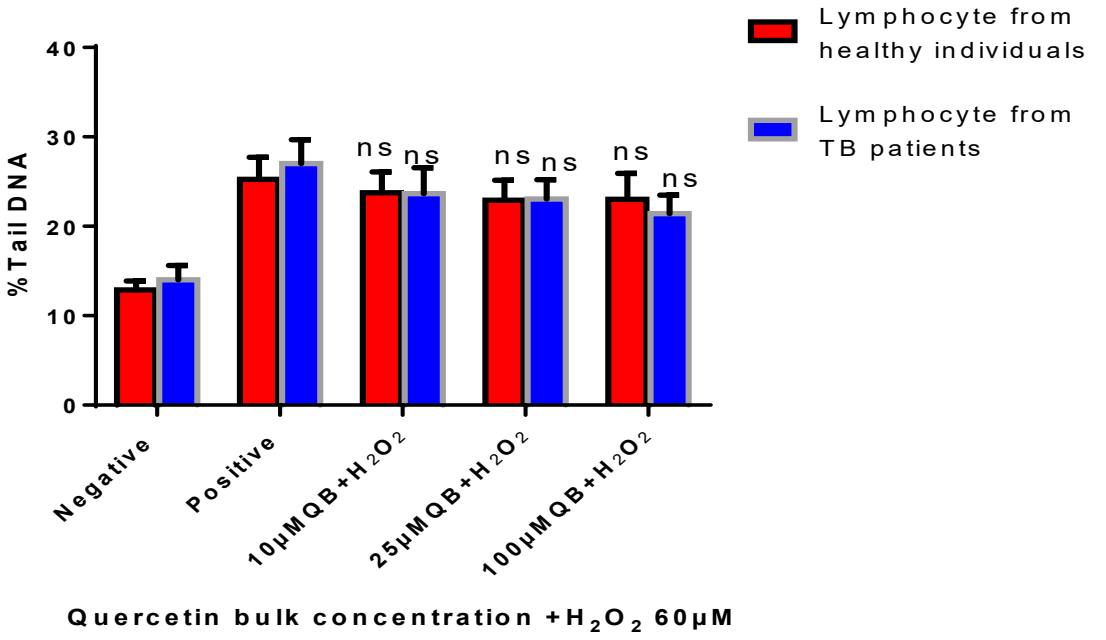


Figure 34: A comparison of % Tail DNA between lymphocytes (figure 18) from 10 different healthy and lymphocytes (figure 22) from 10 TB patients treated with quercetin bulk in the presence of H_2O_2 (60µM), ± SEM Two-Way AVOVA test and significance compared with the positive control (donors=10).

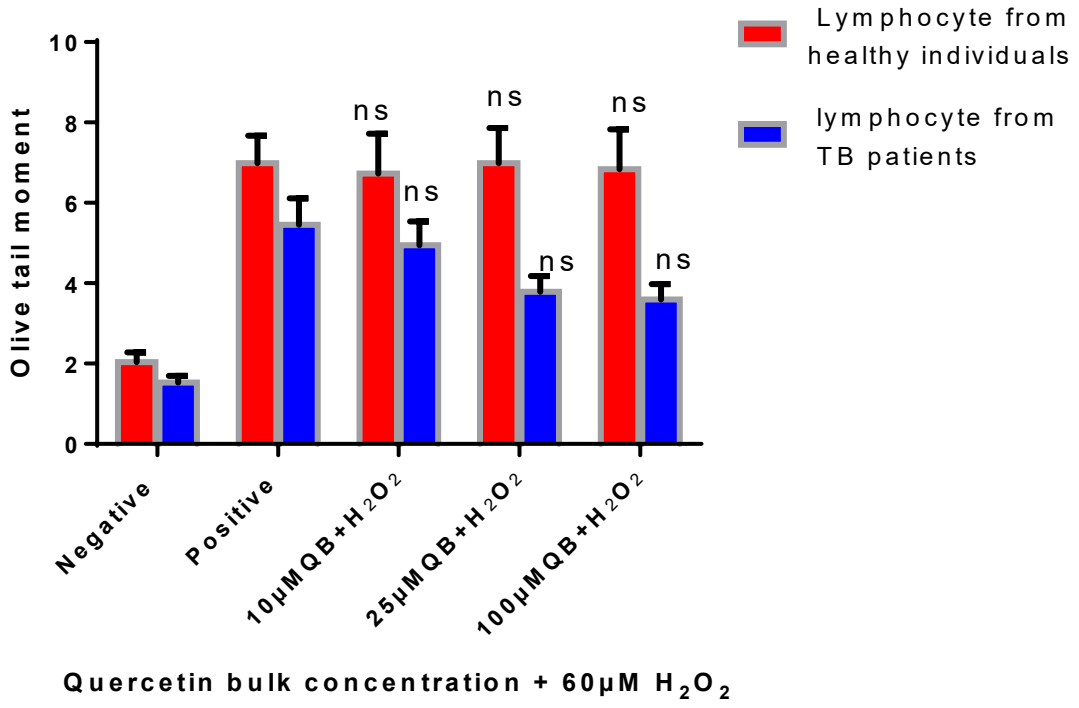


Figure 35: A comparison of Olive tail moment between lymphocytes (figure 19) from 10 healthy individuals and lymphocytes (figure 23) collected from 10 TB patients treated with quercetin bulk in the presence of H_2O_2 (60µM) ± SEM Two-Way AVOVA test and significance compared with the positive control (donors=10).

3.4.15 Comparison of the DNA protective effect of quercetin nano on DNA damage induced by H_2O_2 in lymphocytes collected from healthy individuals and patients with TB.

Figures 36 and 37 display a comparison between lymphocytes collected from TB patients (figure 24 and 25) and lymphocytes collected from healthy individuals (figures 20 and 21) treated with the quercetin nano in the presence of H_2O_2 using % tail DNA and Olive tail moment. It was shown that the influence of quercetin nano was noN-significant in healthy lymphocytes compared to the positive control, while the effect was significant in lymphocytes collected from TB patients where the P value recorded was **P≤0.0120 at a concentration 25 µM and ***P≤0.001 at concentration of 100 µM. The concentration of 10µM of quercetin nano was not significant for % tail DNA and Olive tail moment in the Two-Way ANOVA test.

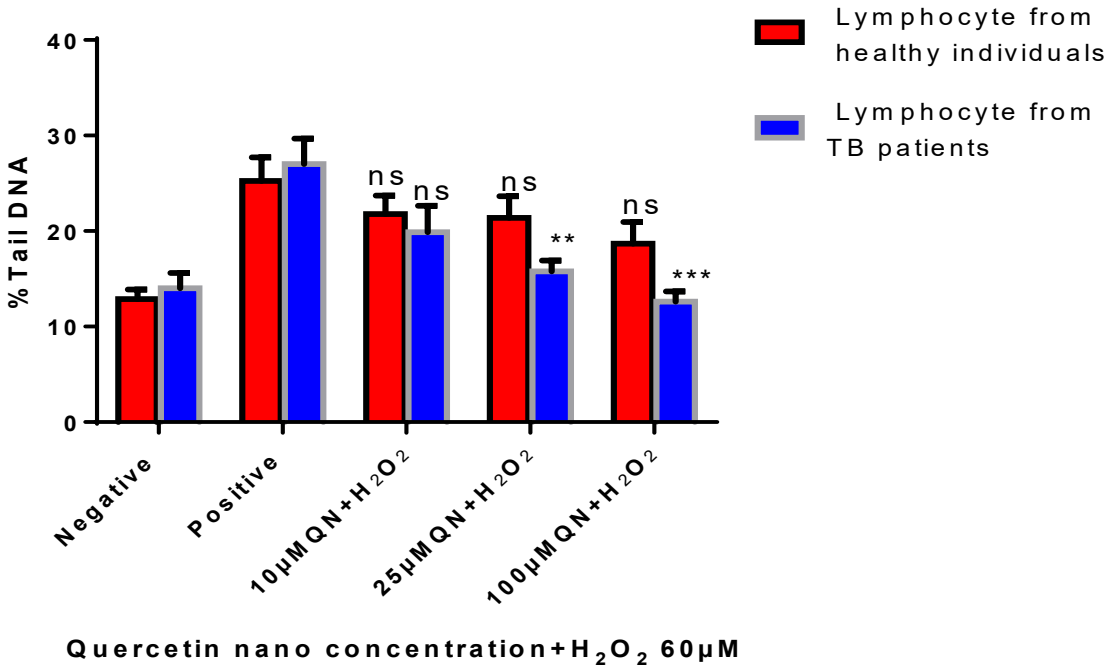


Figure 36 : A comparison of % Tail DNA between lymphocytes (figure 20) from 10 different healthy individuals and lymphocytes (figure 24) collected from 10 patients with TB treated with quercetin nano in the presence of H_2O_2 (60µM), ± SEM Two-Way AVOVA test and significance compared with the positive control (donors=10).

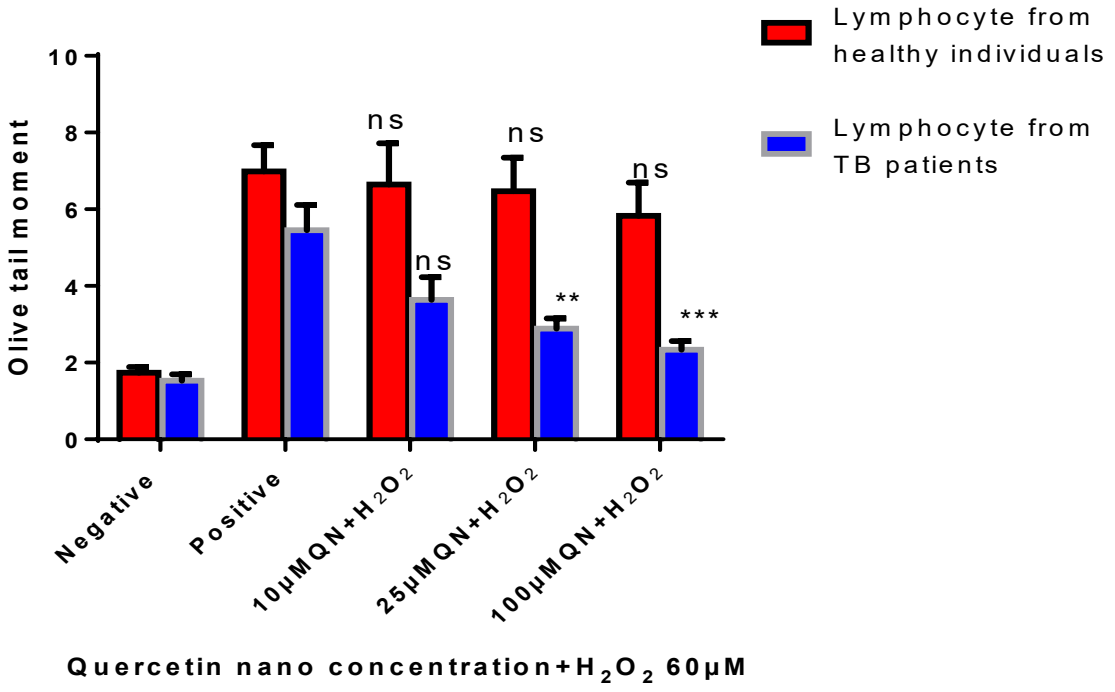


Figure 37: A comparison of Olive tail moment between lymphocytes (figure 21) collected from10 different healthy individuals and lymphocytes (figure 25) collected from 10 patients with TB treated with quercetin nano in the presence of H_2O_2(60µM), ± SEM Two-Way AVOVA test and significance compared with the positive control (donors=10).

3.4.16 Comparison of the DNA protective effect of quercetin bulk and quercetin nano on DNA damage induced by H_2O_2 in lymphocytes collected from patients with TB.

The bar chart 38 and 39 give information about a comparison between bulk (figures 22 and 23) and nano (figures 24 and 25) of quercetin in treated lymphocytes collected from TB patients, compared to the positive control. The % tail DNA showed the effect of the bulk form of quercetin was non-significant at three concentrations 10µM, 25µM, 100µM, whereas the nano form was significant at two concentrations which are 25µM and 100µM. However, the nano form at10µM concentration had a

value of *P*≥0.05 (Figure 33). In figure 33 where the comparison used Olive tail moment, the three concentrations of nano form showed statistically significant reduction of DNA damage in lymphocytes from TB patients in the Two-Way ANOVA test; furthermore, 25µM and 100µM concentrations of the nano form gave the same significance of ****P*≤0.001. However, the 10µM concentration showed a non-significant reduction in DNA damage compared to the positive control. The bulk form did not bring about significant reduction in DNA damage at the lowest concentration of 10µM but was effective in reducing the DNA damage significantly at the higher concentration of 25 µM *P≤0.0024, and 100 µM **P≤0.0014 based on the same statistical analysis.

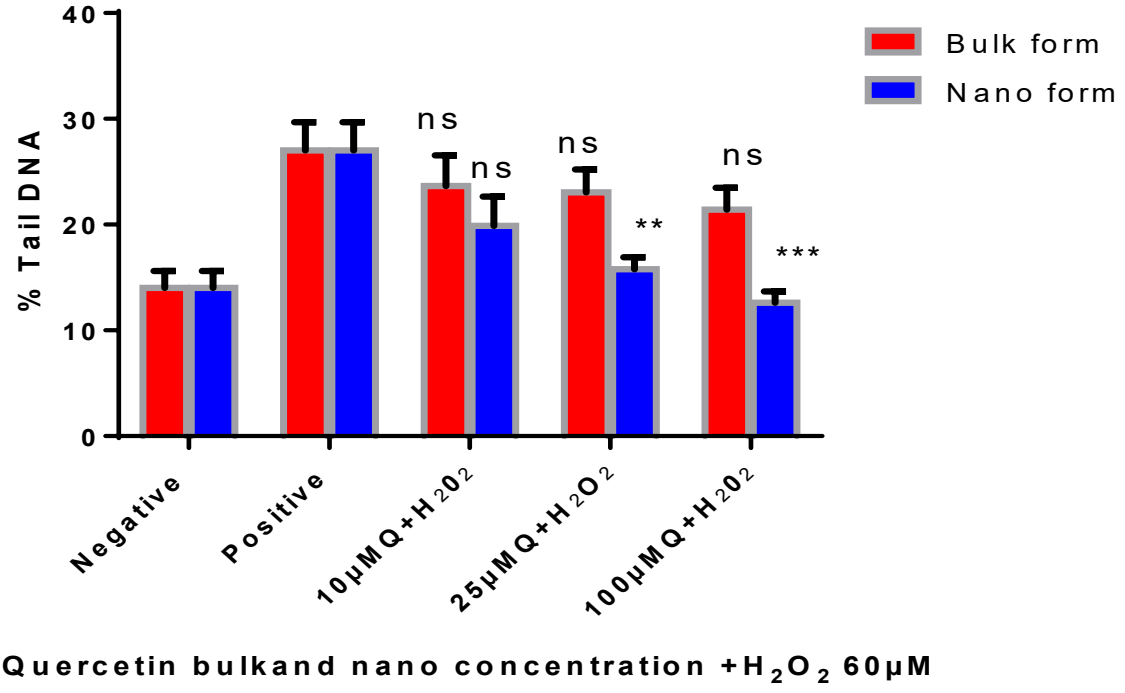


Figure 38: A comparison of % Tail DNA between bulk form (figure 22) and nano form of quercetin (figure 24) treated lymphocytes collected from 10 patients with TB in the presence of H_2O_2 (60µM), ± SEM Two-Way AVOVA test and significance compared with the positive control (donors=10).

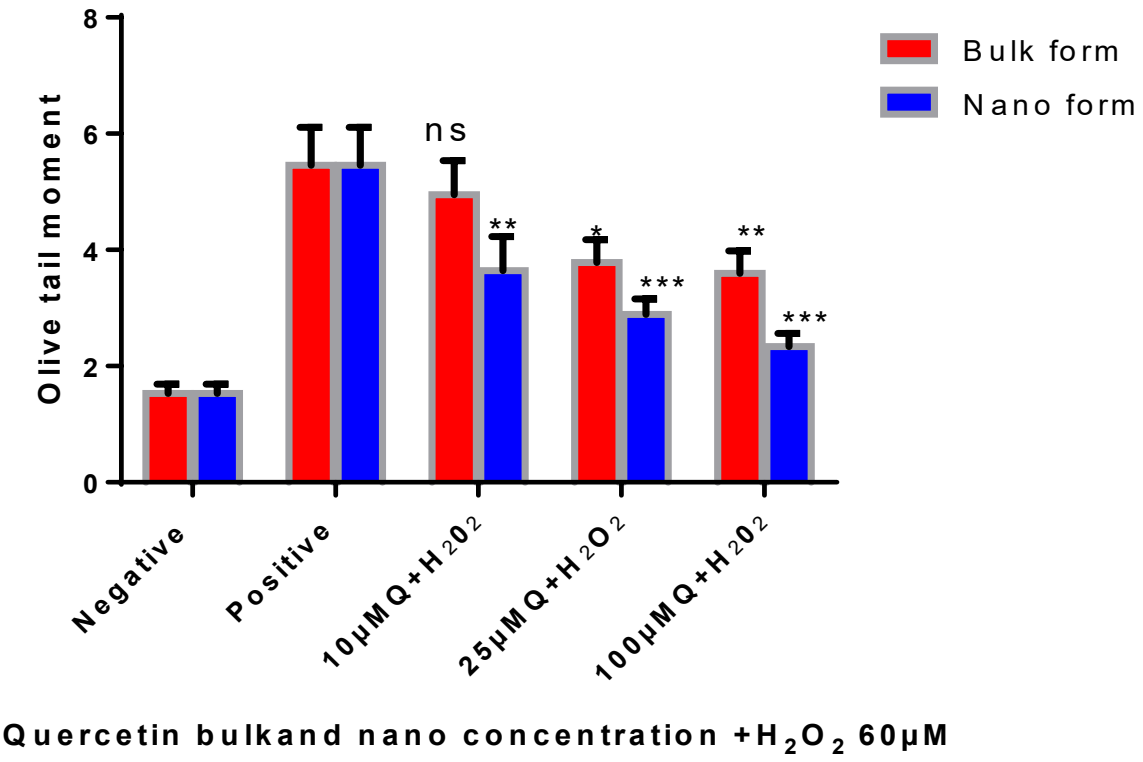


Figure 39: A comparison of Olive tail moment between bulk form (figure 23) and nano form (figure 25) of quercetin treated lymphocytes from 10 patients with TB in the presence of H_2O_2 (60µM), ± SEM Two-Way AVOVA test and significance compared with the positive control (donors=10).

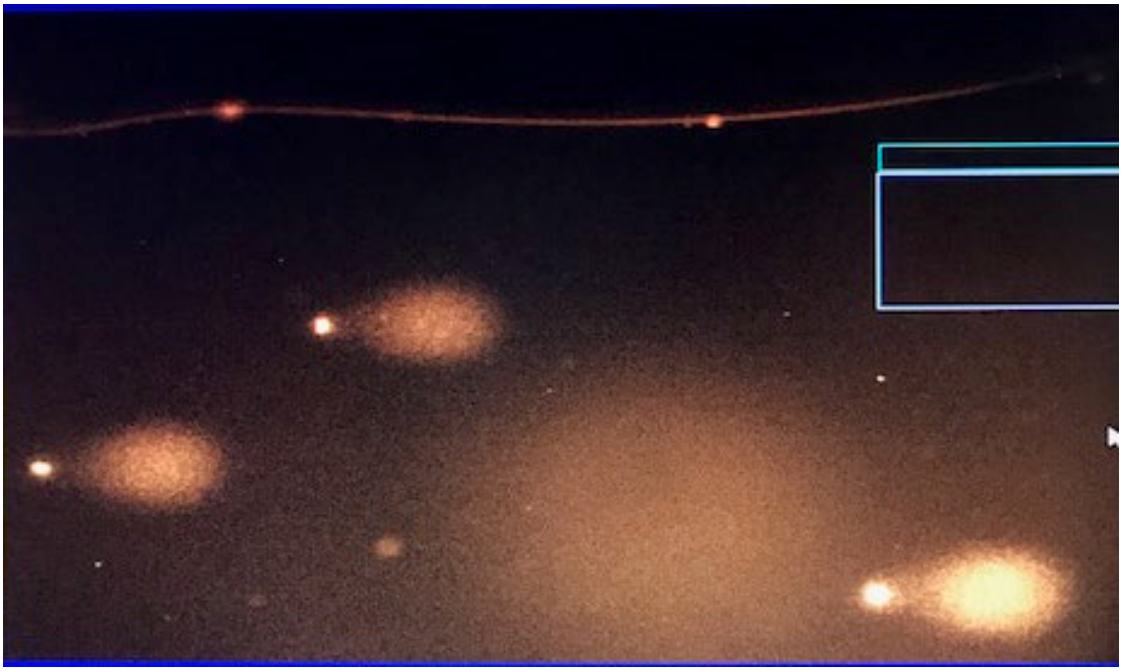


Figure 40: Typical Sperm comet image showing head and tail DNA damage caused by 55µM H_2O_2.

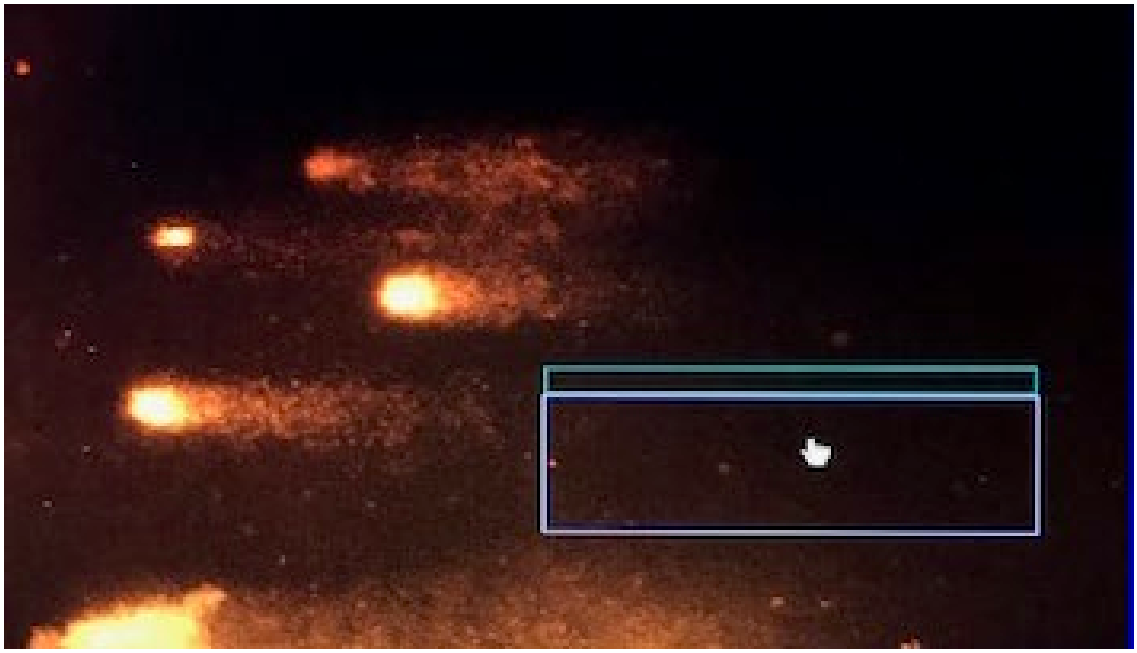


Figure 41: Typical comet image in lymphocytes collected from TB patients showing damage in lymphocytes cell DNA and tail damage caused by 60µM H_2O_2.

3.5 Discussion

In the last two decades, the Comet assay has become one of the most influential and standard assays to evaluate DNA damage and measuring the changes in genomic instability (Anderson et al., 2013). Collins et al (1997) and Wu and Jones (2012) pointed out that the Comet assay is the most sensitive technique to assess DNA strand breakage using neutral or alkaline forms of the assay. The advantages of this method include rapidity, ability to measure DNA damage at the level of a single cell, simplicity and the requirement of small blood samples (Collins et al., 1997, Wu and Jones, 2012). It has been reported that lymphocytes collected from healthy individuals or patients may reflect cancer cells and other disease states to estimate genotoxicity. Furthermore, lymphocytes are suitable for monitoring chromosomal abnormalities, mutations, and sister chromatid exchanges, and after in vitro exposure (Najafzadeh et al., 2012, Anderson et al., 1991). Once a chemical change from the bulk form to the nano form, the surface area of the material is increased, as well as its immunogenicity, drug delivery and solubility due to improved contact between the NPs and the surrounding materials, which increase reactivity (Zhang et al., 2008). The nanoparticles form having a greater surface area than the bulk form, where a greater surface leads to a high opportunity of inflammation in the respiratory system and improved oxidant capacity (Frampton et al., 2004). Nano and bulk forms of quercetin were used to treat ex-vivo sperm and lymphocytes in vitro. Lymphocytes and sperm collected from healthy individuals and lymphocytes obtained from TB patients were treated with three different concentrations of quercetin (10μM, 25 μM and 100 μM) nano and bulk form at the same time. All the experiments were done in vitro in the same settings. The study used H_2O_2 as a positive control, lymphocytes used 60 μM of H_2O_2 as the positive control and 50 μM

of H_2O_2 was used with sperm, this was because sperm are more sensitive and contains half number of chromosomes. Dissimilar results were noticed in the present project between quercetin concentrations and reduction of DNA damage in sperm and peripheral lymphocytes collected from patients with TB from a mean of ten different individuals. Results showed significant protection regarding DNA damage by the nano form compared to the bulk form which treated lymphocytes collected from TB patients (Figures 38 and 39). Using Graph pad prism Two-Way ANOVA test the protection was significant where the P value was ***P≤0.001. While, lymphocytes collected from healthy individuals were induced by 60 μM of H_2O_2 and treated with both forms of quercetin, the effect of quercetin was statistically non-significant (Figures 18, 19, 20, 21). The high response was at 100 μM of quercetin in the nano form where the P value was ***P≤0.001 with both sperm samples and lymphocytes from TB patients (Figures 24, 25 and 28, 29). The reason for this could be that quercetin was more effective as an anti-oxidant compound with a concentration of 100μM. Furthermore, it was shown at 10 μM of quercetin that the nano form was not effective in reducing the DNA damage significantly (Figures 36 and 37, and 17) compared to lymphocytes collected from healthy individuals. In contrast 25 μM of quercetin nano form showed a P value of **P≤0.0026. The highest concentration reduced DNA damage compared to the negative control was 100μM of quercetin nano. The concentrations above 100 μM of quercetin were neglected because recent studies reported that it leads to DNA damage. Moreover, it is difficult to prepare higher concentrations as a nano form (Oztopcu-Vatan et al., 2009). In this present study the results of the Comet assay were compatible with the previous recommendation that flavonoid such as quercetin has antimicrobial resistance for TB and enhance action of the antibiotic drugs (Górniak et al., 2018).

4 Chapter (4) The antigenotoxic effect of quercetin nano and bulk forms in lymphocytes in the micronucleus assay

4.1 Introduction

The Comet assay data from the previous chapter shows that lymphocytes collected from healthy individuals and patients with TB exhibit a noticeable reduction in DNA damage brought about by H_2O_2 treatment in the presence of both the nano and bulk forms of quercetin. The current study employs the micronucleus assay to confirm these findings as this assay is one of the reliable methods of assessing chromosome damage. Both for chromosome loss and chromosome damage (Fenech, 2007). In this technique, genetic instability is measured by analysing the presence of the micronucleus (MN), nucleoplasmic bridges (NPBs), and nuclear buds (NBUDs) in binucleated cells. The assay involves culturing of lymphocytes. The lymphocyte cell cycle takes 24 hours. In the first stage (G1) cells start to produce enzymes that necessary in S phase (Collins et al., 1997b). Chromosomes are replicated in the S phase to yield two copies. Following this, all substances which are required in M phase and help the cell to divide are started by synthesis in the G2 phase (Norbury and Nurse, 1992) Nuclear division is completed in M phase; this stage can be further divided into five different substages starting with prophase, followed by prometaphase, then metaphase, after that anaphase, and finally telophase. The DNA damage is scored in binucleated cells (BiNC) formed due to cytochalasin B (cyto B) stopping cell division at the cytokinesis stage. Genotoxic compounds can cause mutations and DNA damage due to their interfering with chromosomes throughout the cell division (Fenech, 2007). Formation of micronuclei MNi can be created from a fragment or a whole chromosome at the mitosis stage after anaphase. The presence of micronuclei in binucleated cells reflects the presence of chromosome breakage and or whole chromosomes (Fenech, 2007). Nuclear buds NBUDs and Nucleoplasmic bridges (NPBs) which are small bridges linking two

nuclei together are biomarkers of DNA miss-repair and/or telomere end-fusions, and elimination of amplified DNA and/or DNA repair complexes respectively (Fenech, 2007). Furthermore, a dicentric chromosome which occurs as a result of DNA miss-repair is leading to NBUDs which is useful to detect gene amplification. The NBUDs are normally connected to the indigenous nuclei; this is the difference between buds and micronuclei, which are not the only difference. The MNi are separated from both nuclei (Fenech, 2007, Luzhna et al., 2013). The Micronucleus assay has been chosen to examine the effect of quercetin nano and bulk forms in treated lymphocyte cells.

4.2 Materials and Methods

4.2.1 Materials

All chemicals and reagents are already shown in chapter 2 table (1) page number 47

4.2.2 Methods

Fresh blood was used in this technique; the collection of blood samples from healthy individuals and patients is as reported in chapter 2.5. page 50. The protocol of this assay is described in chapter 2 section 2.11 (page numbers 56-59). DNA damage was induced in lymphocytes using Bleomycin and treated with different concentrations of nano and bulk forms of quercetin as shown in chapter 2, table 5- page number 57. Examination of lymphocytes collected from healthy individuals and lymphocytes collected from patients with TB were repeated three times to confirm the results depending on MNi frequency and other cytogenetic parameters.

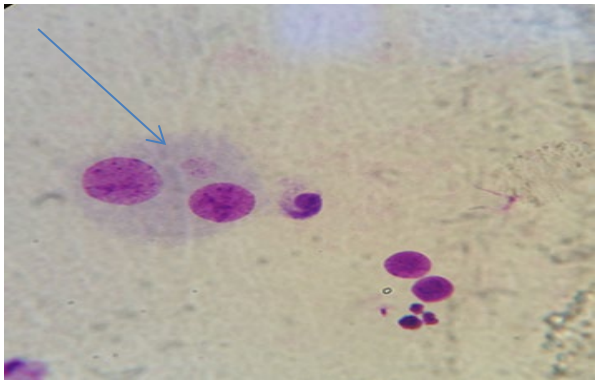


Figure 42: Bi-nucleated cells with one micronucleus.

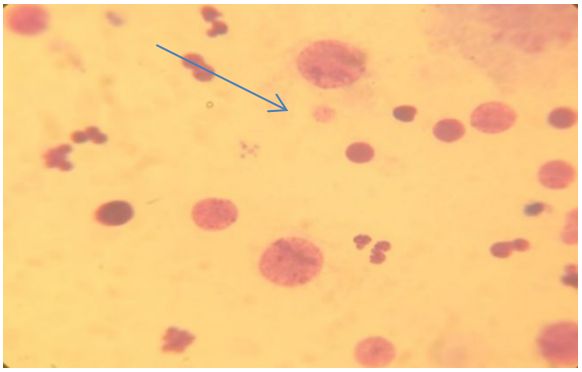


Figure 43: Mono-nucleated cells with one micronucleus.

Three concentrations of quercetin were used in this technique 10µM, 25 µM and 100 µM with both untreated cells and positive control which was 0.4 µM of mitomycin c. In this assay, two slides were made for each concentration, 500 cells were scored for each slide under a light microscope to detect MNi, NBUDs, and NBPs. Cells were calculated to determine the percent of MonoNC, BiNC, and MultiNC. The Nuclear

index division (NDI) can be calculated using three cell types; (NDI) can reflect the rate of mitotic division (Fenech, 2007).

4.2.3 Statistical Analysis

All the results were obtained by investigating 500 cells for each slide and at every concentration there was a need to score two slides as per the Fenech protocol (Fenech, 2007). The (NDI) was determined for each slide and compared with the result obtained for bleomycin. The Graph pad prism T test two tail and two-way ANOVA test were used for the analysis of the results.

4.3 Micronucleus results

4.3.1 Bleomycin concentration response results:

Bleomycin is a natural glycopeptide used as an anticancer treatment; at the same time, it can cause DNA damage due to free radicals that are released. Micronucleus can be formed due to the effect of bleomycin, Nucleoplasmic bridges, and nuclear buds reflect DNA damage in cancer and normal cells (Dedon and Goldberg, 1992). For this reason, five different concentrations of bleomycin (0.55, 0.65, 0.75, 0.85, 0.95µg/ml) were chosen to treat lymphocytes collected from three different healthy individuals to identify the concentration causing the highest damage. Table 9 and figure 44 display the mean of the results from lymphocytes of three healthy individuals treated with five different concentrations of bleomycin. The total cytogenetic damage that included (MNi, NPBs, and buds mean) described the influence of bleomycin on lymphocytes collected from healthy individuals where the NDI rate was between 1 - 1.5 and bi-nucleated cells occupied 30% to 45%. Different cytological scoring parameters were expressed as a percentage out of all types of 500 cells scored. It is clear from the bar chart (figure 44) that the concentration of 0.75µg/ ml produced the highest damage according to One-Way ANOVA test results with a significance value ***P≤0.001 compared to the negative control. The concentration 0.65µg/ml displayed significance *P≤ 0.027 and other concentrations exhibited non-significant levels of DNA damage. Based on these results, the concentration of 0.75µg/ml was chosen as the most appropriate concentration to cause DNA damage in this study.

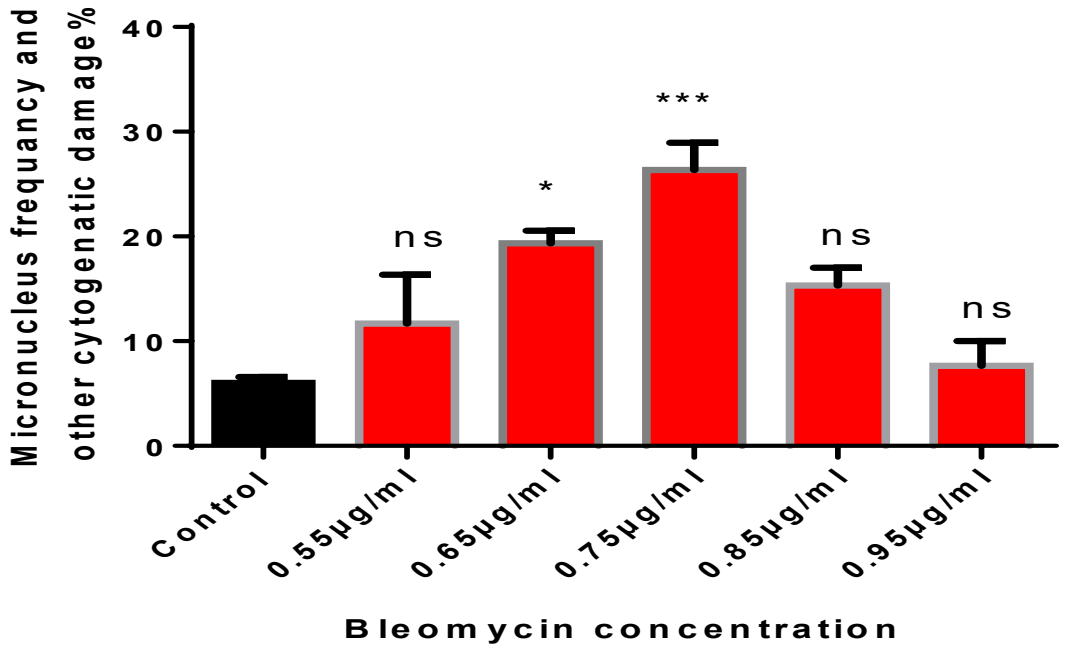


Figure 44: Concentration response of lymphocytes from three different healthy individuals treated with range of concentrations of bleomycin, ± SEM One-Way AVOVA test and significance compared with the control (donors=3).

Bleomycin	Negative	0.55µg/ml	0.65µg/ml	0.75µg/ml	0.85µg/ml	0.95µg/ml
Mon %	52.00	60.00	60.80	64.70	68.80	84.00
Bio %	45.00	35.70	35.70	31.40	30.80	30.60
NID	1.50	1.43	1.42	1.41	1.34	1.16
MNi mean	1.00	4.30	7.30	11.30	6.30	1.00
Buds mean	3.00	2.00	7.60	9.00	5.00	3.60
NPBS mean	2.00	5.30	4.30	6.60	4.00	3.30
Total cytogenetics damage	6.00	11.60	19.30	26.30	15.30	7.60

Table 8: Summary of micronucleus test on lymphocytes from three different healthy individuals treated with range concentrations of bleomycin. The results expressed over 1000 cells each concentration scored (donors=3).

4.3.2 A comparison of the genoprotective effect of bulk and nano forms of quercetin on lymphocytes from healthy individuals treated with bleomycin using micronucleus assay

Bar chart 45 and table 10 provide information on the genoprotective effect of bulk and nano form of quercetin on lymphocytes treated with bleomycin using the micronucleus assay. Lymphocytes were collected from healthy individuals incubated with bleomycin and treated with the two forms of quercetin separately. It is obvious from the bar graph 45 and table 10 that both forms of quercetin brought about reduction in the percentage of micronuclei compared to bleomycin (0.75µ/ml). The only concentration that was non-significant is the 10µM quercetin bulk form where the P value was greater than P≥ 0.05, while the concentration of 100µM of the bulk and nano forms of quercetin showed a significant reduction in the formation of micronuclei, statistical significance based on Two-Way ANOVA test ****P*≤ 0.001compared to the bleomycin.

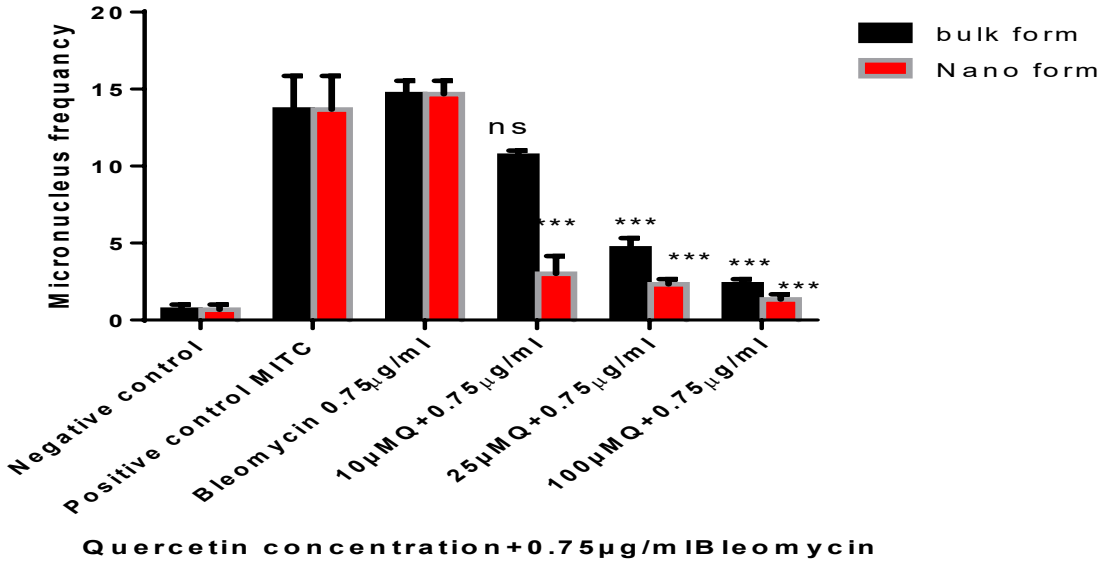


Figure 45: A Comparison between bulk and nano form of quercetin based on MN frequencies in lymphocytes collected from three different healthy individuals treated with quercetin bulk and nano forms, ± SEM T test two tail and significance compared with the bleomycin (donors=3).

File	NC	PC	Bleomycin	10μMQB	10μMQN	25μMQB	25μMQN	100μMQB	100μMQN
Mon%	50.50	59.00	53.00	55.00	58.50	57.00	55.00	55.50	55.30
Bio %	44.00	34.20	44.00	39.00	36.50	45.00	41.00	40.90	41.30
Multi %	3.80	4.80	7.30	4.80	3.70	3.10	3.30	3.20	3.10
NID	1.50	1.50	1.50	1.50	1.40	1.47	1.49	1.47	1.48
MNi	1	15	15.5	9.5	2	5	2	2	1
Bio-Buds	6	10	8.5	9.5	11	11.5	8	4.5	2
Bio-NPBS	1	4	3.5	6	1	4	1	1.5	1
Total cytogenetic damage	8.00	29.00	27.50	24.00	14.00	20.50	11.00	8.00	4.00

Table9: Summary of different cytological parameters from the micronucleus assay of lymphocytes from healthy individuals treated with bulk and nano forms of quercetin represented by Mon-nucleated%, Bi-nucleated %, nuclear division index, MNi micronucleus cells, Bi-nucleated Buds, Bi-NPBs and total cytogenetics damage. The results expressed over 1000 cells each concentration scored (donors=3).

4.3.3 A comparison of the genoprotective effect of bulk and nano forms of quercetin on lymphocytes from TB patients treated with bleomycin using micronucleus assay

Bar chart 46 and table 11 illustrates the comparative genoprotective effect of bulk and nano form of quercetin in bleomycin treated lymphocytes collected from TB patients. Overall, the effect of the nano form was more significant than the bulk form based on the comparison with bleomycin using a Two-Way ANOVA test. The first concentration of the bulk form reduced micronucleus formation partially, and statistically was significant at *P≤ 0.0281. However, the other two concentrations of bulk and the concentration 10 μM of the nano form were shown the same significance and the P value gives **P≤ 0.027. The higher effect of quercetin was with the nano form with two concentrations 25 μM and 100μM where the significance was ***P≤ 0.001.

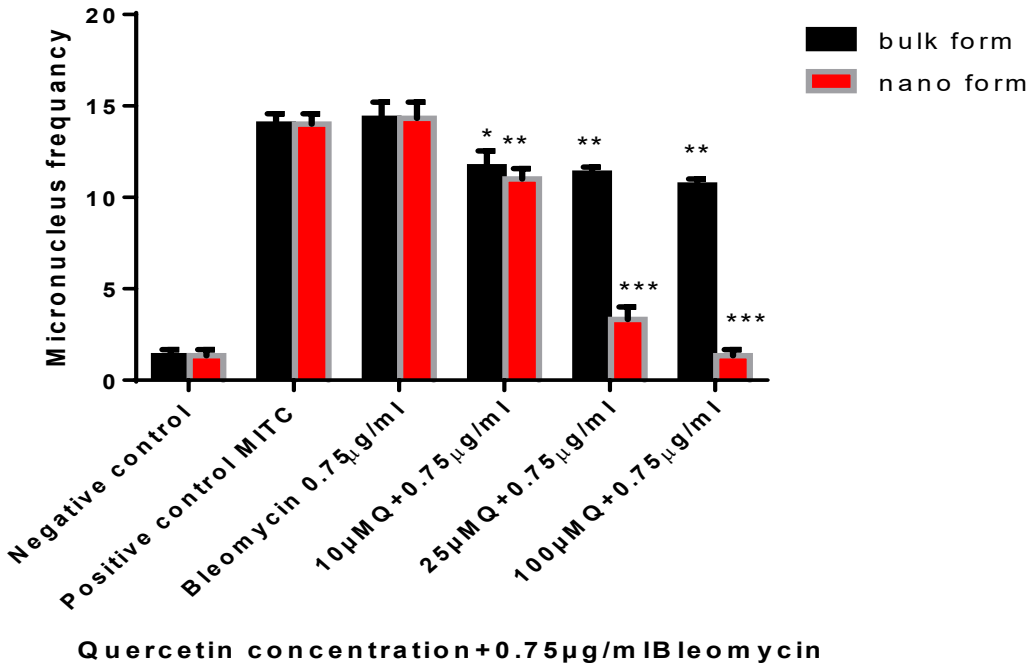


Figure 46: A Comparison between MN frequency in lymphocytes collected from three different patient with TB treated with quercetin bulk and nano form in the presence of 0.75µg/ml of bleomycin. The results expressed over 1000 cells each concentration scored including ± SEM and significance level using T test two tail (donors=3).

File	NC	PC	Bleomycin	10µMQB	10µMQN	25µMQB	25µMQN	100µMQB	100µMQN
Mon%	53.80	53.00	52.20	60.20	61.00	64.00	59.60	57.50	56.00
Bio %	43.50	41.00	44.20	38.40	35.80	37.80	40.10	36.70	41.00
Multi %	2.7.00	6.00	3.6.00	1.40	3.20	1.00	1.00	6.50	3.00
NID	1.47	1.48	1.48	1.39	1.48	1.38	1.40	1.40	1.43
MNi	1.3	14	14.3	11.6	11	11.3	3.3	10.6	1.3
Bio-Buds	5.3	7	12	12.6	6.3	10.6	9	7.6	4.3
Bio-NPBS	4.3	4.3	5	4.6	4	3.6	4	4	2.6
Total cytogenetic damage	10.60	25.30	31.30	27.50	17.60	23.50	16.30	17.90	8.20

Table 10: Summary of MN frequencies in lymphocytes collected from healthy individuals treated with the bulk and nano forms of quercetin represented by Mono-nucleated%, Bi-nucleated %, nuclear division index, MNi micronucleus cells, Bi-nucleated Buds, Bi-NPBs and total cytogenetics damage. The results expressed over 1000 cells each concentration scored (donors=3).

4.3.4 A comparison of the ability of quercetin bulk form in reduction of micronucleus induction in lymphocytes from healthy individuals and lymphocytes from TB patients treated with bleomycin

Bar graph 47 shows a comparison of the genoprotective effect of the bulk form of quercetin in terms of percentage reduction in the micronucleus in lymphocytes from healthy and lymphocytes from patients with TB treated bleomycin. It is clear from the bar chart that the decrease in the rate of micronuclei by quercetin bulk in lymphocytes collected from healthy individuals was similar to lymphocytes collected from TB patients. At 10μM concentration, the bulk form of quercetin shows no significant effect with the Two-Way ANOVA test. The similarity of the effect was clear at 25μM where the statistical analysis confirmed identical levels of significance; it recorded P *P≤0.0283. The highest reduction for the micronucleus rate was observed with 100μM of the bulk form of quercetin treated lymphocytes collected from patients with TB which showed a significance level of **P≤0.0271.

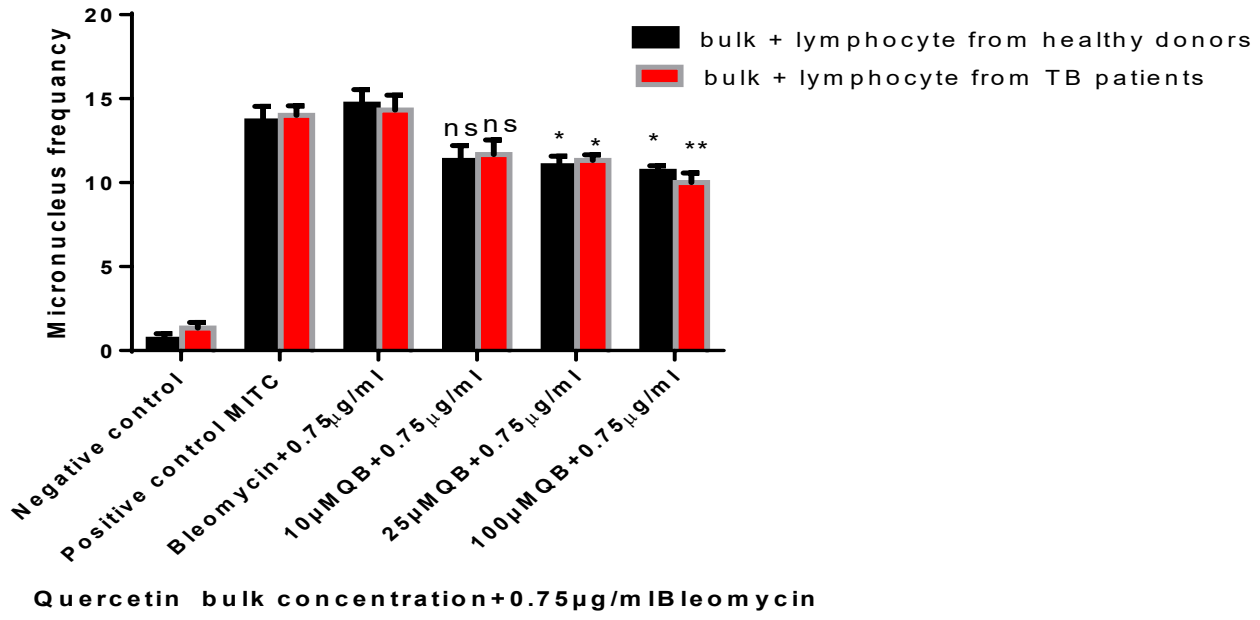


Figure 47 : A comparison of MN frequencies in lymphocytes from three different healthy individuals and lymphocytes from three TB patients treated with quercetin bulk in the presence of 0.75μg/ml of bleomycin including standard errors and significance using T test two tail. The results expressed over 1000 cells each concentration scored (donors=3).

4.3.5 A comparison of the ability of quercetin in the nano form in reducing micronucleus induction in lymphocytes from healthy individuals and lymphocytes from TB patients treated with bleomycin

Bar chart 48 gives information about the ability of the quercetin nano form in reducing the induction of MNi in lymphocytes from healthy individuals compared to lymphocytes from patients with TB treated with 0.75µg/ml bleomycin. It is clear from figure 48 that the rate of micronucleus induction was significantly reduced when treated with 25 µM and 100µM of quercetin nano in lymphocytes from healthy individuals and also those from patients with TB. The Two-Way ANOVA test revealed significance of ***P≤0.001 with lymphocytes of both healthy individuals and TB patients. The decrease of MNi was less significant at 10µM where the P value showed **P≤0.0271) with healthy individuals. However, the lowest significance was with lymphocytes collected from TB patients and shown *P≤0.0283.

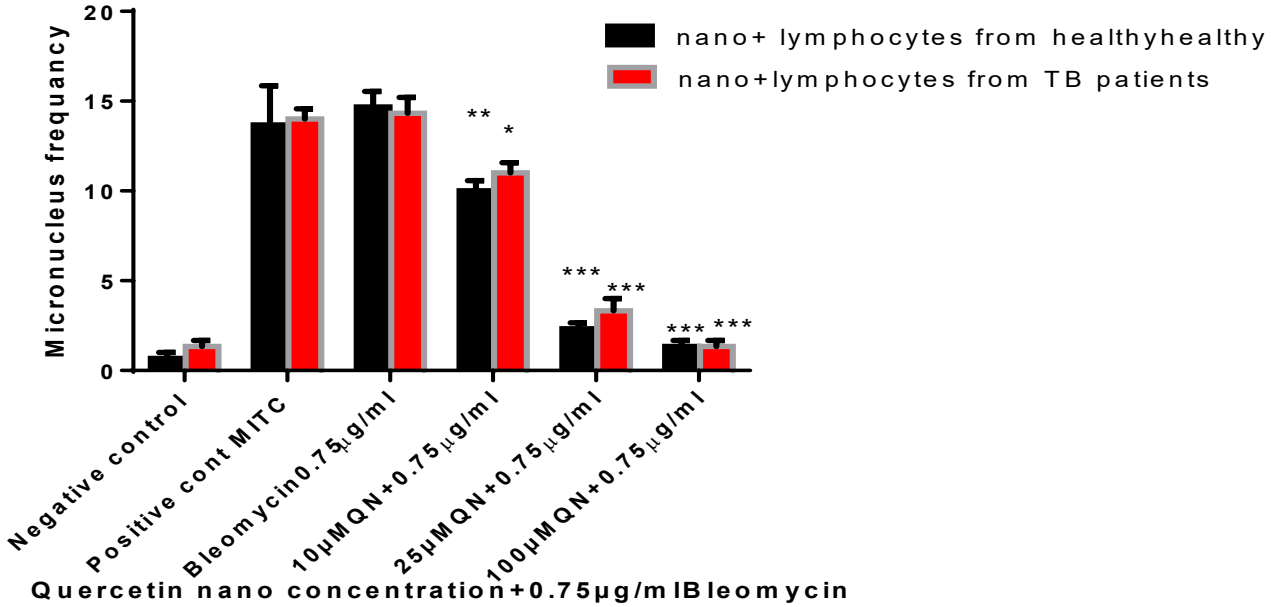


Figure 48: A Comparison between MNi frequency in lymphocytes from three patients with TB and three lymphocytes from healthy individuals treated with quercetin bulk in the presence of 0.75µg/ml bleomycin including standard errors and significance level using T test two tail. The results expressed over 1000 cells each concentration scored (donors=3).

4.4 Discussion

Micronuclei (MNi) are formed during anaphase of the nuclear division. It consists of a small, whole chromosome or a fragment of the chromosome which lags behind during anaphase and is not joined with the daughter nucleus (Fenech, 2007). The MNi were discovered over 100 years ago by Howell and Jolly in the cytoplasm of erythrocytes and the haematologist named it the “Howell-Jolly bodies” (Kirsch-Volders et al., 2003). Later Fenech used Cytochalasin-B (Cyt-B) to block cytokinesis resulting in the development of the Cytokinesis-block Micronucleus (CBMN) assay. This technique is an inexpensive and reliable technique for scoring DNA damage, micronucleus rate (MNi), nuclear buds (NBUDS) and nucleoplasmic bridges (NPBs). Moreover, it can evaluate the percentage of mono-, bi-, and multinucleated cells, the effect of cytotoxicity and measuring the rate of apoptotic and necrotic cells (Albertini et al., 2000). The reduction of MNi by bulk and nano form of quercetin as the protective compound in the bio-nucleated lymphocytes was investigated using this technique. Many studies have approved that regular intake of flavonoids like quercetin and rutin play a major role in cancer prevention (Li et al., 2001). Results from tables (10, 11) illustrated a normal value of NDI rated between (1.3 - 1.5). The vast majority of lymphocytes had completed one cell division. 1% - 6% of lymphocytes from TB patients, and 3% - 7% of lymphocytes from healthy individuals were multinucleated. This is proof that a low percentage of lymphocytes have started the second cell division (mitosis) in the presence of cyto-B. Different results were obtained from lymphocytes collected from healthy individuals and TB patients where the MNi production compared to bleomycin using both forms of quercetin. The positive control and the negative control in all experiments were normally distributed. The bulk size of quercetin at a concentration of 10μM did not significantly reduce the

micronucleus frequency in both lymphocytes collected from healthy individuals and patients with TB (figure 47), whereas the nano form was significant at the same concentration *P≤0.0283 bar graph 48. The best reduction of MNi rate was at 100μM of quercetin in nano form the significance was ***P≤0.001 compared with bleomycin bar graph 48. Comparing two forms of quercetin (bulk and nano); the result of the comparison indicated that the nano form has a significant influence on MNi frequency more than the bulk size. In 2009 Singh et al, reported the difference in these forms. They pointed out that, nanoparticles have a greater ability to penetrate the nucleus membrane than the bulk size (Singh et al., 2009). MNi are assumed to reflect the primary stage in development of genomic instability in TB patients. Therefore, reducing MN frequency by quercetin in lymphocytes from TB patients was applicable (Luzhna et al.,2013). Moreover, *Mycobacteria* can stimulate reactive oxygen species (ROS) production by activating phagocytes where hydrogen peroxide (H_2O_2) is released. The H_2O_2 is accumulated constantly by the macrophages and a resulting high free radical load in pulmonary cells in TB patients which may lead to cancer development, this condition like bleomycin effect that releasing free radicals, then oxidative stress that casing DNA damage (Adebimpeet al., 2016). Results of this assay suggest that a small dose of quercetin could have a therapeutic role as a protective compound with bleomycin to decrease the level of DNA damage among patients with TB.

5 Chapter (5) The influence of quercetin on the level of DNA damage caused by heterocyclic amines PhIP and IQ in lymphocytes and Sperm

5.1 Introduction

Heterocyclic amines (HCA) have been recognised as having an important effect on human health and can raise the risk of cancer (Emmons et al., 2005). Many studies reported that colorectal cancer relates to HCA exposure (Knize et al., 1995). The HCA can be formed during cooking proteinaceous food; basically, through the heating effect on free amino acids, monosaccharides, and creatinine. Moreover, HCA can be a causal factor of mutagenic action in the bacterial test systems such as Ames/Salmonella assay where the methyl groups' location can cause the mutagenicity (Felton and Knize, 1991). The heterocyclic amines can be distinguished into three different derivatives: amino-imidazo-pyridines (e.g. PhIP), amino-imidazo-quinolines (e.g. IQ) and amino-imidazoquinoxalines (e.g. 2-amino-3, 8-dimethylimidazo [4,5 –f]quinoxaline, 8-MeIQx). The PhIP seemed to be less effective in causing DNA damage when examined in bacterial cells (Pfau et al., 1999). Anderson et al. (2001) reported that the lymphocytes collected from patients diagnosed with a disease state are more sensitive to DNA damaging agents compared with those from healthy individuals (Anderson et al., 2001; Najafzadeh et al., 2009).

In the current study the food mutagens, IQ and PhIP were used to induce DNA damage in lymphocytes collected from healthy individuals and patients with TB in the presence of different concentrations of bulk and nano forms of quercetin. The level of DNA damage was measured using the Comet assay to evaluate the genoprotective effect of quercetin.

5.2 Materials and methods

The materials and methods, as well as the experimental design, have been described in Chapter 2 section 2.12.6-page number 59.

5.3 Statistical analysis

Data obtained as % Tail DNA and Olive tail moment were analysed by Graph pad prism 6 and SPSS for Windows, statistical package (version 18.0). The data were tested for normal distribution, using Kolmogorov-Smirnoff and Shapiro Wilk's test. One-Way ANOVA test was used to examine the significance of experimental results obtained for the lymphocytes collected from healthy individuals and patients with TB. All the results were compared with IQ or PhIP samples in the presence of quercetin of different concentrations. Moreover, the two forms bulk and nano of quercetin were compared with each other using the same parameters.

5.4 Results

5.4.1 Concentration response experiments on the IQ induced DNA damage in lymphocytes from healthy individuals induced by IQ at different concentrations.

Figures 49 and 50 provide information on the concentration response of IQ induced DNA damage in lymphocytes collected from healthy individuals. It is clear from the histograms that the DNA damage reaches a peak at 150μM and 175μM of IQ in both parameters, % Tail DNA and Olive tail moment. Graph pad One-Way ANOVA test showed high significance **P≤0.0043 at 150μM and 175μM using %Tail DNA the concentrations were compared to the negative control. The trend was dropped at 200μM in the two parameters. Based on these results, the concentration of 160μM was chosen as a concentration of IQ used in the experiments.

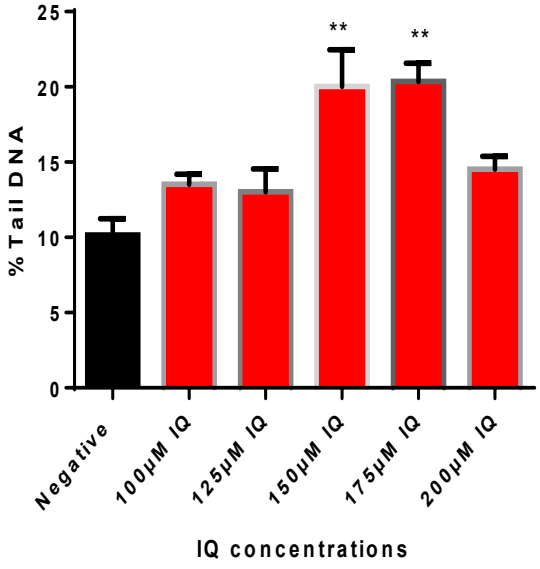


Figure 49: Different concentrations of IQ treated lymphocyte collected from three different healthy individuals presented as means of the response of % Tail DNA, ± SEM, One-Way AVOVA test and significance compared with the untreated cells (donors=3).

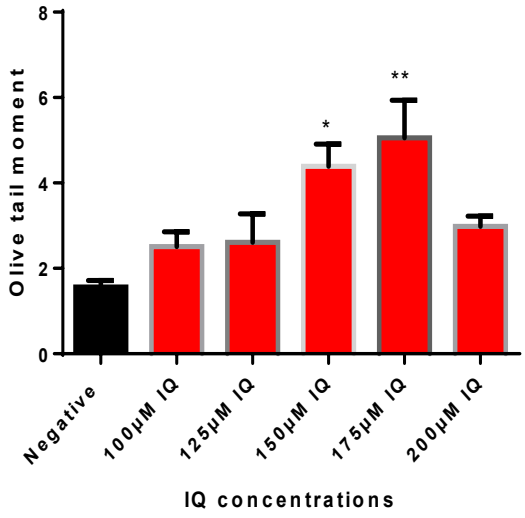


Figure 50: Different concentrations of IQ treated lymphocyte collected from three different healthy individuals presented as means of the response of Olive tail moment, ± SEM, One-Way AVOVA test and significance compared with the untreated cells (donors=3).

5.4.2 The effect of quercetin bulk and nano forms on DNA damage induced by 160µM of IQ in lymphocytes collected from healthy individuals.

Figures 51 and 52 illustrate data related to lymphocytes collected from healthy individuals treated with quercetin bulk and nano forms in the presence of IQ at 160µM. On the basis of % Tail DNA, it is clear that both the forms of quercetin can reduce DNA damage. The reduction was statistically significant and rated between **p ≤ 0.0043 to ***p ≤ 0.001 depending One-Way ANOVA test, whereas all the concentrations of the bulk form were non-significant for Olive tail moment. All treatments were compared to lymphocytes with IQ only.

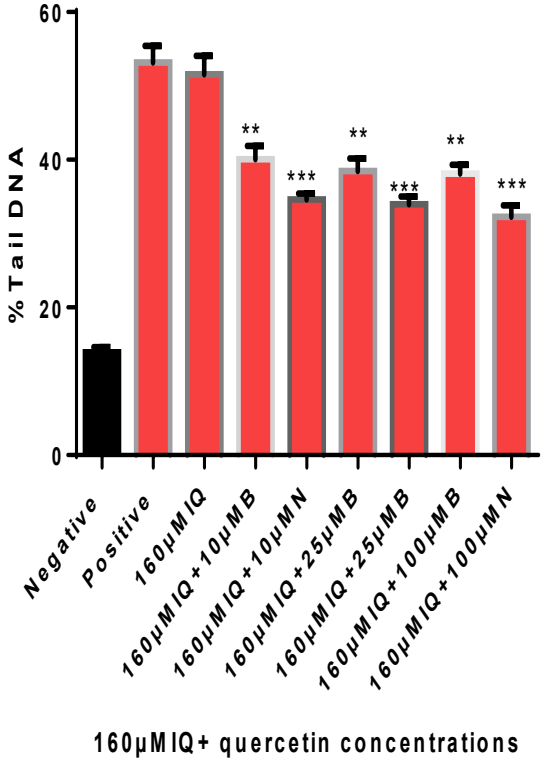


Figure 51: The effect of quercetin bulk and nano forms on DNA damage presented as the means of % tail DNA of lymphocyte collected from 10 different healthy individuals treated with quercetin at different concentrations in the presence of IQ 160µM ± SEM, One-Way AVOVA test and significance compared with 160 µM of IQ (donors=10).

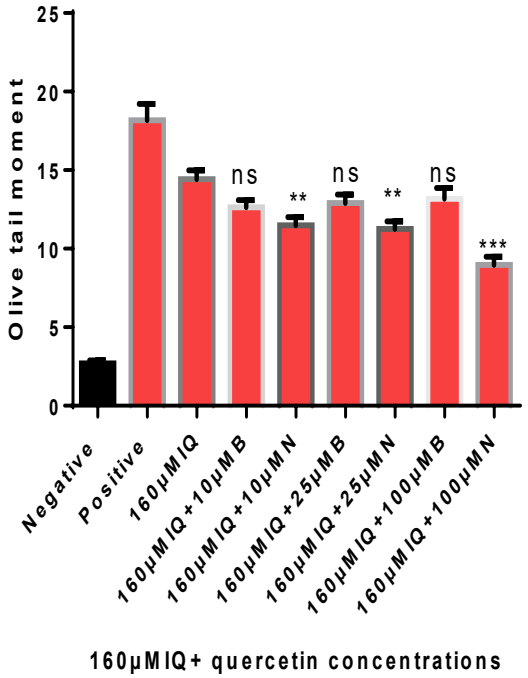


Figure 52: The effect of quercetin bulk and nano forms on DNA damage presented as the means of Olive tail moment of lymphocyte collected from 10 different healthy individuals treated with quercetin different concentrations in the presence of IQ 160µM ±SEM,One-Way AVOVA test significance compar to 160 µM IQ (donord=10)

5.4.3 Effect of bulk and nano forms of quercetin on DNA damage induced by 160μM of IQ in lymphocytes collected from TB patients.

Figures 53 and 54 show information related to lymphocytes collected from TB patients treated with quercetin bulk and nano forms in the presence of IQ 160μM. The most striking features of the two bar charts are that the quercetin has the potential to reduce DNA damage caused by IQ. The term % Tail DNA illustrates a significant reduction where the P value ranged between**p ≤ 0.0043 to ***p ≤ 0.001. However, the results of the Olive tail moment were different; the bulk form was significant only at 100μM and *p ≤ 0.0405. The maximum reduction in DNA damage was observed with 100μM of the nano form. All treatments were compared to lymphocyte samples treated with IQ only.

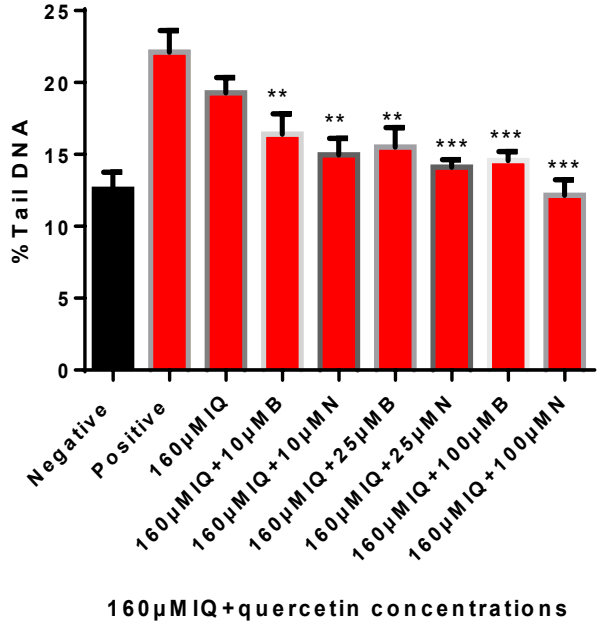


Figure 53: The effect of quercetin bulk and nano forms on DNA damage presented as the means of % Tail DNA of lymphocyte collected from 10 TB patients treated with quercetin different concentrations in the presence of IQ 160μM, ±SEM,One-Way AVOVA, significance compared with the 160 μM of IQ (donors=10).

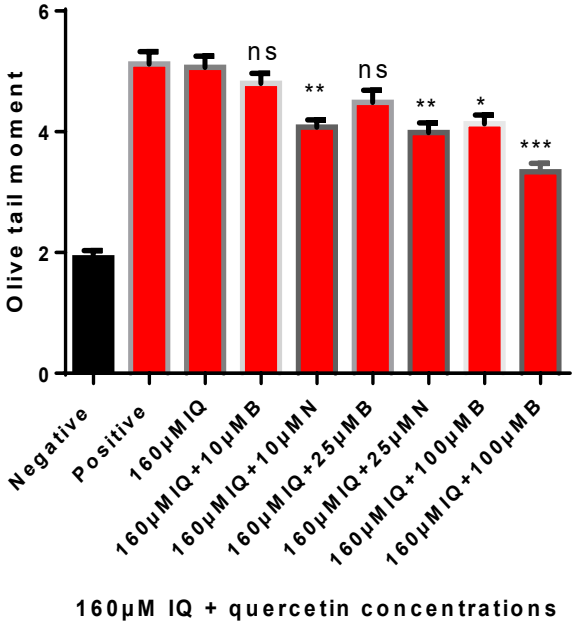


Figure 54: The effect of quercetin bulk and nano forms on DNA damage presented as the means of Olive tail moment of lymphocyte collected from TB patients treated with quercetin different concentrations in the presence of IQ 160μM, ±SEM,One-Way AVOVA, significance compared with the 160 μM of IQ (donors=10).

5.4.4 Dose response experiments with sperm from healthy individuals induced by IQ concentrations.

Figures 55 and 56 provide the concentration response of sperm samples from healthy individuals treated with IQ. It is evident from the results (Figures 55 and 56) that the concentrations 125μM and 150μM exhibit the highest DNA damage. However, the damage at other concentrations is non-significant or less significant both in terms of % tail DNA and Olive tail moment. All the concentrations were compared with the negative control. Based on one-way ANOVA test, the negative control, and all concentrations were normally distributed.

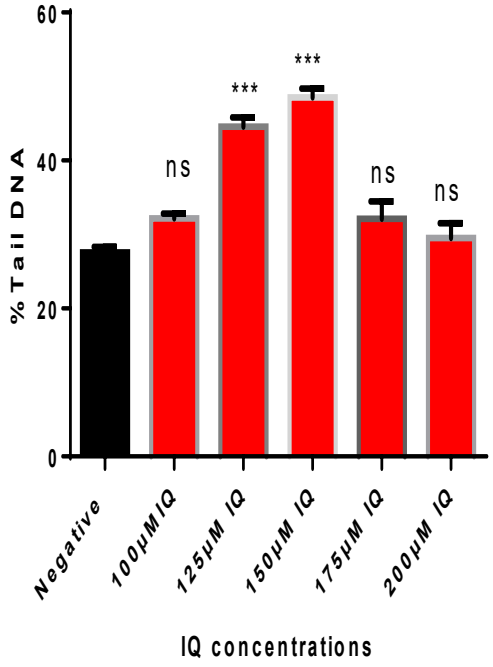


Figure 55: Different concentrations of IQ treated sperm from three different healthy individuals presented as the response means of % Tail DNA, ±SEM,One-Way AVOVA test significace compared with the untreated cells, (donors=3).

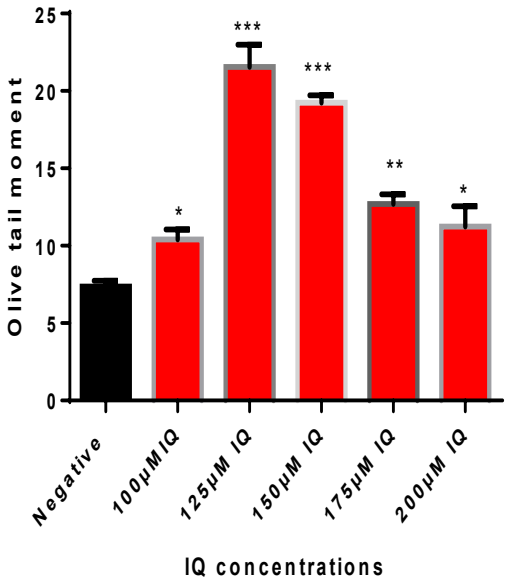


Figure 56: Different concentrations of IQ treated sperm from three different healthy individuals presented as the response means of Olive tail moment, ±SEM,One-Way AVOVA test significace compared with the untreated cells (donors=3).

5.4.5 Effect of quercetin bulk and nano forms on DNA damage induced by 140µM of IQ in sperm collected from healthy individuals.

Figures 57 and 58 illustrate data on the ability of the two forms of quercetin in reducing DNA damage induced by IQ 140µM in sperm collected from healthy individuals. It was apparent from % Tail DNA and Olive tail moment; that the nano form of quercetin significantly reduced the damage to sperm DNA at 25µM and 100µM where the p-value rated between *p ≤ 0.043 and ***p ≤ 0.001. There was a non-significant DNA damage with a bulk form of quercetin based on One-Way ANOVA test. All concentrations were compared to sperm sample treated with IQ only. The negative control, positive control (50µM H_2O_2), IQ and quercetin concentrations were distributed normally.

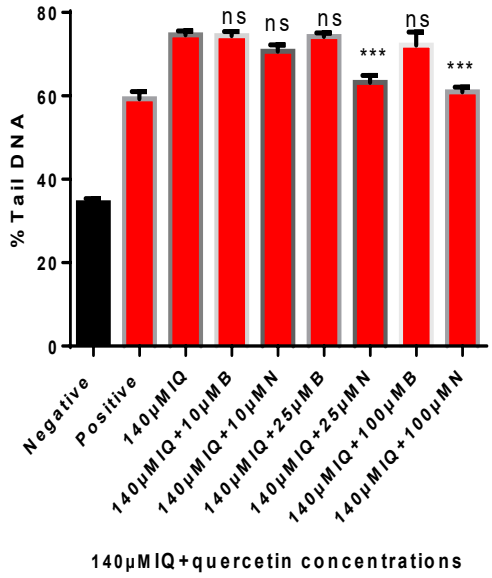


Figure 57: The effect of quercetin bulk and nano forms on DNA damage presented the means of % Tail DNA of sperm collected from 10 different healthy individuals induced by 140µMIQ, ±SEM, One-Way AVOVA test significace compared with the 140µM of IQ (donors=10).

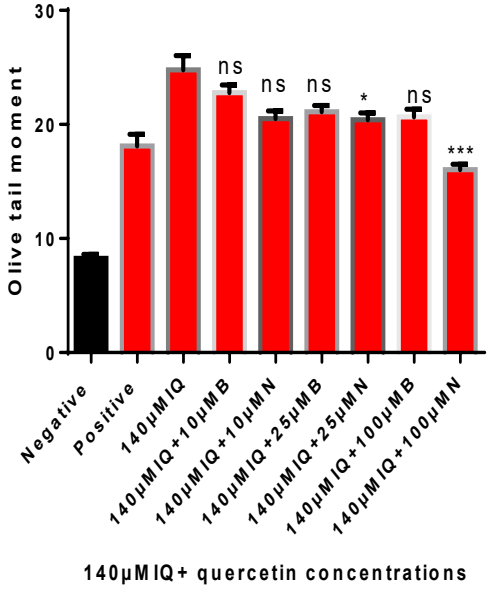


Figure 58: The effect of quercetin bulk and nano forms on DNA damage presented as the means of Olive tail moment of sperm collected from 10 different healthy individuals induced by 140µMIQ, ±SEM,One-Way AVOVA test significace compared with the 140µM of IQ (donors=10).

5.4.6 Concentration response of PhIP induced DNA damage in lymphocytes from healthy individuals.

Figures 59 and 60 are display information about the concentration response of lymphocytes collected from healthy individuals to PhIP. It is clear that from both histograms that the DNA damage reached the peak at 150μM of PhIP in both terms of % Tail DNA and Olive tail moment, the statistical analysis using a Graph Pad One-way ANOVA test showing the highest significance at 150 μM where the ***P≤0.001. All the concentrations were compared to the negative control. The trend was dropped at 175μM in both parameters. Based on these results the concentration 140μM of PhIP was chosen as a concentration used for experiments involving PhIP on lymphocytes.

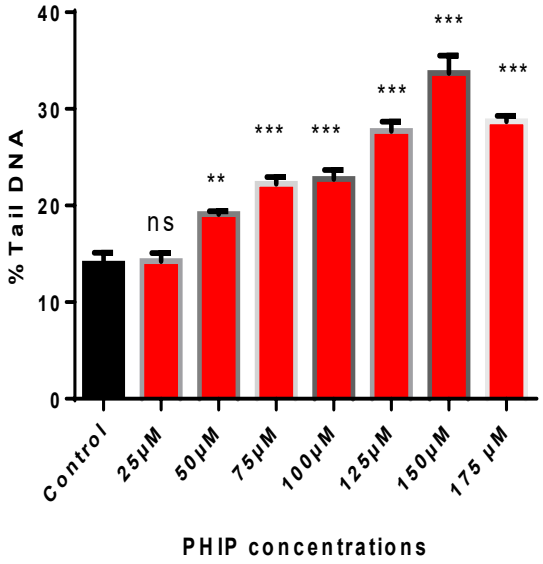


Figure 59: Different concentrations of PhIP presented as the means of % Tail DNA of lymphocytes collected from three different healthy individuals, ±SEM,One-Way AVOVA test, significace compared with the untreated cells (donors=3).

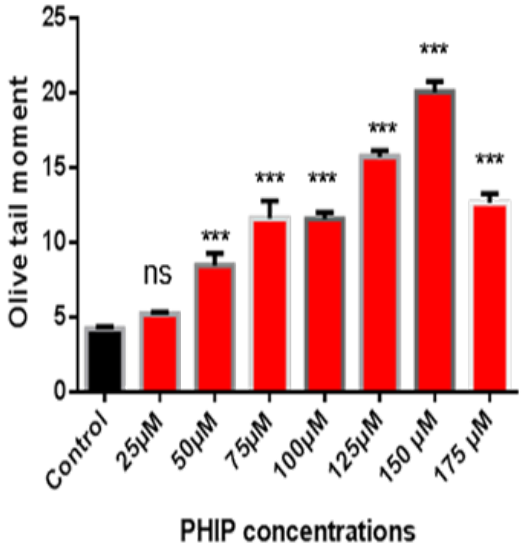


Figure 60: Different concentrations of PhIP presented as the means of Olive tail moment of lymphocytes collected from three different healthy individuals, ±SEM,One-Way AVOVA test significace compared with the untreated cells (donors=3).

5.4.7 Effect of quercetin bulk and nano forms on DNA damage induced by 140µM of PhIP in lymphocytes collected from healthy individuals.

Figures 61 and 62 illustrate information about effect of bulk and nano forms of quercetin on lymphocytes from healthy individuals treated with PhIP 140µM. The experiment show that two forms of quercetin brought about a reduction in DNA damage expressed in terms of both % Tail DNA and Olive tail moment. The reduction was statistically significant and rated between *p ≤ 0.0043 and ***p ≤ 0.001 depending on the One-Way ANOVA test. The best DNA reduction was at 100µM of quercetin, while 10µM of quercetin bulk showed *p ≤ 0.0043. All the treatments were compared to lymphocyte treated with PhIP only. The negative control, positive control (60µM H_2O_2) and quercetin concentrations were normally distributed.

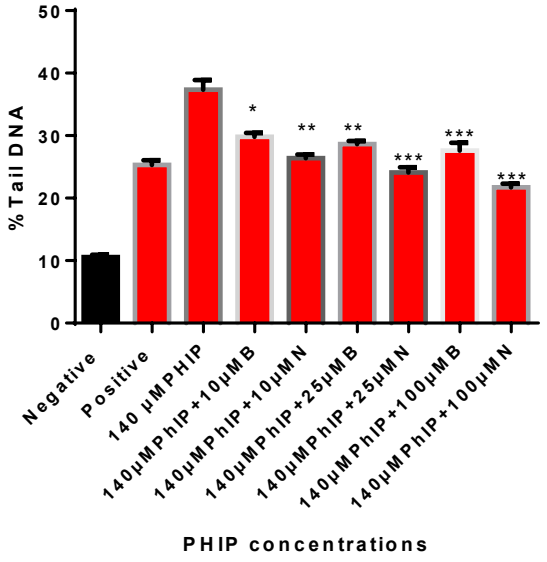


Figure 61: The effect of quercetin bulk and nano forms on DNA damage presented as the means of % Tail DNA of lymphocytes from 10 different healthy individuals induced by PhIP140µM, ±SEM,One-Way AVOVA test significace compared with the 140µM PhIP (donors=10).

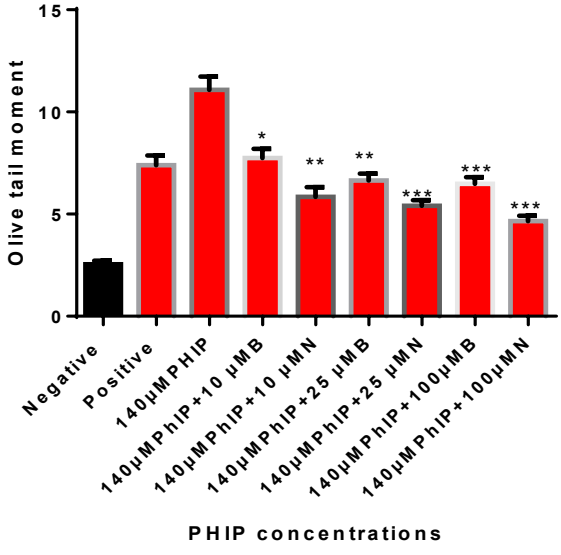


Figure 62: The effect of quercetin bulk and nano forms on DNA damage presented as the means of Olive tail moment of lymphocytes from 10 different healthy individuals induced by PhIP140µM, ±SEM,One-Way AVOVA test, significace compared with the 140µM PhIP (donors=10).

5.4.8 Effect of quercetin bulk and nano forms on DNA damage induced by 140µM of PhIP in lymphocytes collected from patients with TB.

Figure 63 and 64 demonstrate data of the genoprotective ability of bulk and nano forms of quercetin in lymphocytes collected from TB patients treated with 140µM of PhIP. It was clear that both forms of quercetin significantly reduced the damage in lymphocyte DNA collected from TB patients compared to PhIP. The reducing was rated between *p ≤ 0.0043 and ***p ≤ 0.001 based on the One-Way ANOVA test using both parameters % tail DNA and Olive tail moment. All quercetin concentrations were compared to PhIP only. The negative control, positive control which is 60µM of H_2O_2 and quercetin concentrations were normally distributed.

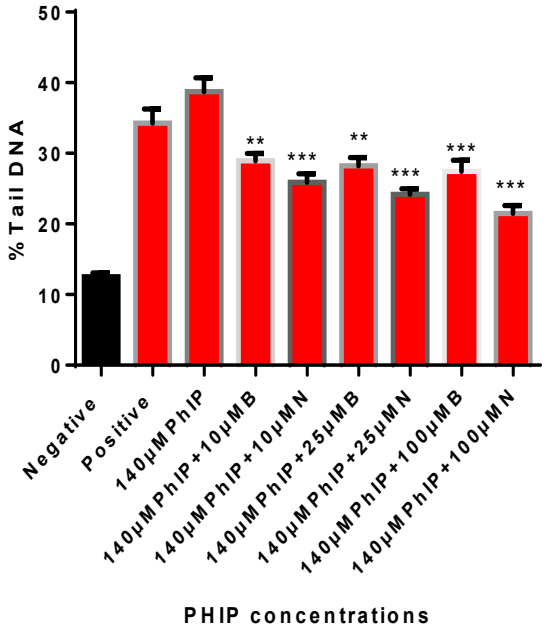


Figure 63: The effect of quercetin bulk and nano forms on DNA damage presented as the means of % Tail DNA of lymphocytes collected from 10 TB patients induced by 140µM of PhIP, ±SEM,One-Way AVOVA test, significace compared with the 140µM PhIP (donors=10).

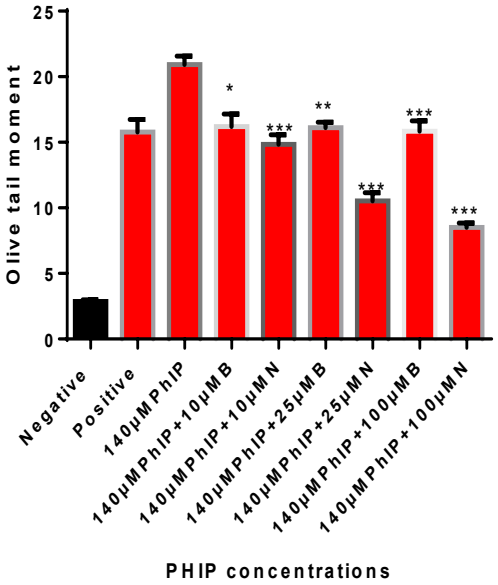


Figure 64: The effect of quercetin bulk and nano forms on DNA damage presented as the means of Olive tail moment of lymphocytes collected from 10 TB patients induced by 140µM of PhIP, ±SEM,One-Way AVOVA test, significace compared with the 140µM PhIP (donors=10).

5.4.9 Concentration response of PhIP induced DNA damage in sperm from healthy individuals

Figures 65 and 66 give information about damage induced by different concentrations of PhIP on sperm collected from healthy individuals treated with different concentrations of PhIP to determine the concentration response using % Tail DNA and Olive tail moment. It was evident from figure 65 and 66 that the concentrations 100µM and 125µM of PhIP caused the highest damage in sperm DNA compared to negative control. The comparison used the One-Way ANOVA test and showed ***p ≤ 0.001 for both concentrations. The concentrations 25µM, 150µM, 175µM showed a non-significant DNA damage compared to negative control.

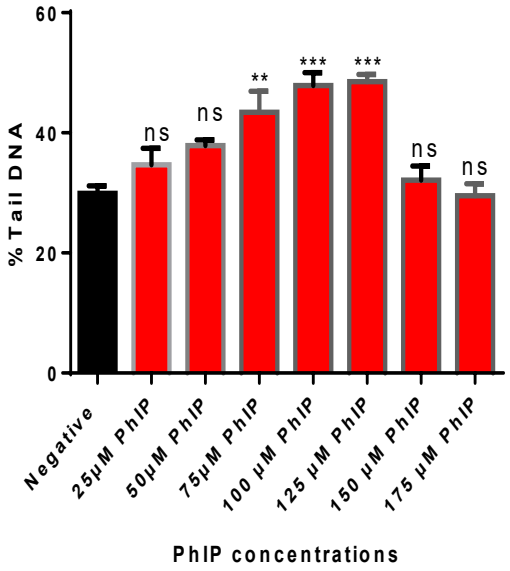


Figure 65: Different concentrations of PhIP presented as the response for means of % Tail DNA of sperm collected from three different healthy individuals, ±SEM,One-Way AVOVA test significace compared with the untreated cells (donors=3).

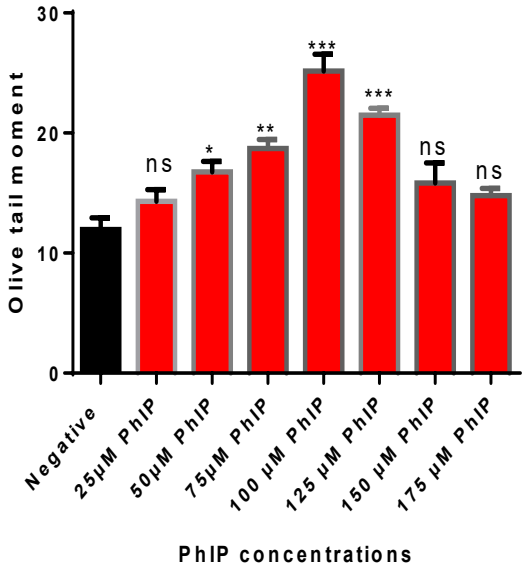


Figure 66: Different concentrations of PhIP presented as the response for means of Olive tail moment of sperm collected from three different healthy individuals, ±SEM,One-Way AVOVA test significace compared with the untreated cells (donors=3).

5.4.10 Effect of bulk and nano forms of quercetin on DNA damage induced by 100µM of PhIP in sperm collected from healthy individuals.

The Figures 67 and 68 illustrate the genoprotective effect of the two forms of quercetin in sperm from healthy individuals treated with 100µM of PhIP. It is clear from figures 67 and 68 that the effect of quercetin on sperm DNA was significant at some concentrations, the 10µM of quercetin bulk in both parameters was non-significant compared to PhIP. It was clear that higher concentrations of both forms of quercetin significantly reduced the damage in sperm DNA compared to samples treated with PhIP only. The reduction was rated between *p ≤ 0.0043 and ***p ≤ 0.001 based on the one-way ANOVA test. The greater DNA damage protection was at 100µM of quercetin nano.

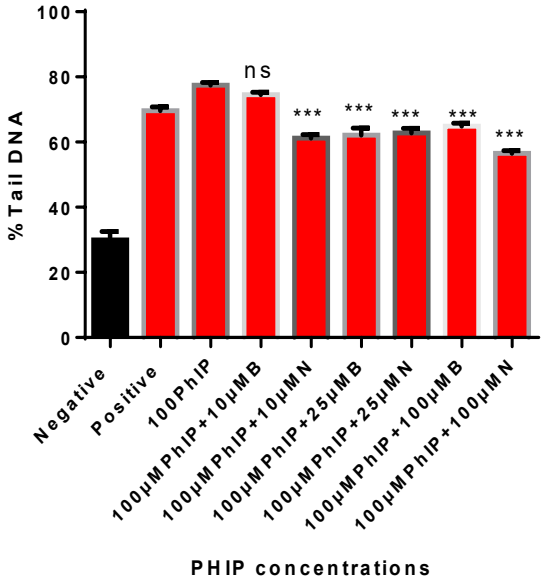


Figure 67: The effect of bulk and nano forms of quercetin on DNA damage presented as the means of % Tail DNA of sperm collected from 10 different healthy individuals induced by 100µM of PhIP, ±SEM,One-Way AVOVA test, significace compared with the 100µM of PhIP (donors=10).

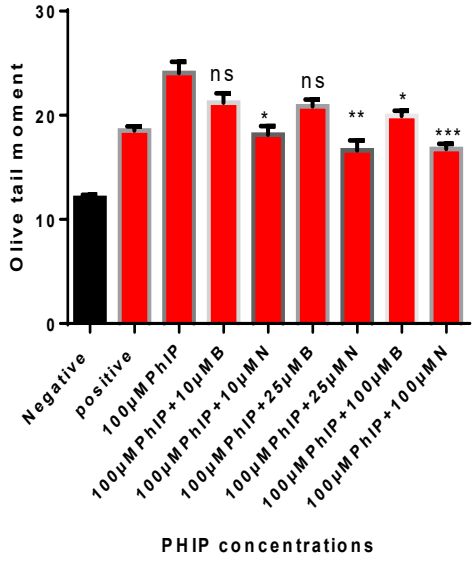


Figure 68: The effect of bulk and nano forms of quercetin on DNA damage presented as the means of Olive tail moment of sperm collected from 10 different healthy individuals induced by 100µM of PhIP, ±SEM,One-Way AVOVA test, significace compared with the 100µM of PhIP (donors=10)

5.5 Discussion

The damaging influences of both food mutagens, IQ and PhIP on the DNA was investigated in this chapter, lymphocytes, and sperm collected from healthy individuals and lymphocytes from patients with TB where treated *in vitro* to evaluate the ability of quercetin to reduce DNA damage. A comparison of the responses of lymphocytes and rectal cells by Pool-Zobel et al. (2004) have revealed a good correlation of the extent of DNA damage on exposure to genotoxic compounds in these two types of cells. These results support our protocol that uses the lymphocytes as mirror cells for the study of cancer and other disease states. The two food mutagens PhIP and IQ were considered to be carcinogenic and genotoxic (Durling and Abramsson-Zetterberg., 2005). Felton et al, (2004) reported that PhIP can develop carcinogenicity through DNA damage or oestrogen receptors while IQ has the potential to form DNA adducts like, N-(deoxyguanosine-8-yl)-IQ in the presence of nitric oxide that instituting a possible cancer risk for patients who are diagnosed with colon inflammation (Lakshmi et al., 2008). Also, HCA can cause DNA damage through the reactive intermediates, which is another possible mechanism to induce-DNA damage; the superoxide anion can be produced when food mutagens like IQ are incubated in the presence of NADH (Maeda et at., 1999).

In this present study, the Comet assay was used to assess DNA damage due to oxidative stress induced by food mutagens IQ and PhIP and this is because the Comet assay in recent years has becomes a technique used for direct assessment of genotoxic DNA damage in lymphocytes and sperm. Furthermore, the Comet assay has become one of the most important assays for measuring the changes in genomic stability and detecting DNA damage (Anderson et al., 2013). Collins in 1997 and Wu, Jones in 2012 have also pointed out that the Comet assay is the most

sensitive assay to study DNA strand breaks in the alkaline or neutral versions at present. The advantages of the Comet assay were linked to the time of the experiment, reasonable cost, simplicity, high sensitivity, and only small blood samples were needed (Kapka-Skrzypczak et al., 2011). Different sensitivities of lymphocytes and sperm samples were assessed. The samples were collected from ten different healthy individuals as well as ten patients with TB. The lymphocytes collected from healthy individuals had DNA induced damage by IQ 160μM,125 μM and PhIP140 μM, 100μM, these concentrations were considered to cause the highest damage for lymphocytes and sperm respectively without toxicity (Henderson, Albertini and Aardema, 2000). Figures (49, 50,55,56,59,60,65 and 66), the sperm and lymphocytes were treated with both nano and bulk forms of quercetin in the same time at different concentrations (10μM, 25 μM, and 100 μM). All the experiments were completed *in vitro* in the same settings. Results indicated significant protection regarding DNA damage by quercetin in both forms that treated lymphocytes collected from TB patients (figures 53 and 54), sperm collected from healthy individuals (figures 57 and 58) and lymphocytes collected from healthy individuals (figures 51 and 52). Using a One-Way ANOVA test the protection was significant and ***P value was (≤0.001). There was a non-significant DNA reduction in effect using the bulk form of quercetin treated sperm collected from healthy individuals (figures 55 and 56). The results were confirmed by two parameters % Tail DNA and Olive tail moment (Figures 57 and 58) the concentration 140μM of PhIP was chosen as a concentration that caused the highest DNA damage of lymphocytes without toxicity (Henderson, Albertini and Aardema, 2000). Both forms of quercetin were seen to reduce the DNA damage in lymphocytes induced by 140μM of PhIP; the cells were collected from healthy individuals and TB patients, and % Tail DNA

and Olive tail moment were used to evaluate DNA damage reduction. The reduction was significant and the *p*-value were rated between *$p \leq 0.0043$ and ***$p \leq 0.001$based on the One-Way ANOVA test figures (59, 60, 61, and 62), whereas sperm collected from healthy individuals was induced by 100μM of PhIP depending on (figures 55 and 56); quercetin was reduced sperm DNA damage that was induced by PhIP more efficiently compared to IQ (figures 55, 56, 65 and 66). The positive and negative controls in all experiments highlighted as anticipated. The results have suggested that a small concentration of quercetin nano has a protective action to reduce DNA damage caused by heterocyclic amines in patient with TB. Vitaglione and Fogliano, (2004) reported that antioxidants can be used to decrease the human health risk associated with heterocyclic amines in food (Vitaglione and Fogliano, 2004).

6 Chapter (6) Investigation of expression of COX1, COX2, P53 and Bcl-2 transcripts in lymphocytes collected from healthy individuals and patients with TB

6.1 QPCR technique to investigate the expression of COX1, COX2, P53 and Bcl-2 in lymphocytes from healthy individuals and patient with TB.

6.2 Introduction

Real-time (RT-) PCR is a method that is commonly used to study gene expression, where quantity of DNA, cDNA, or RNA (PCR products) to be measured is small. The RT-PCR protocol relies on the detection of fluorescence formed by the amplification of the target molecules. This fluorescent target molecule contains dyes that connect to the DNA strand or sequence-specific investigations. RT-PCR has many advantages, such as quickness, the accuracy of results, and a high rate of sensitivity. Another advantage of this technique, which is very important, is that it has a low rate of contamination caused by PCR products (cDNA or RNA) (Klein, 2002). RT-PCR requires expensive reagents and understanding the experimental design of this technique is very crucial, as it is a susceptible procedure (Wong and Medrano, 2005). The different PCR types are powerful techniques that are used widely in genotoxicity and gene expression. Gao et al., (2014) pointed out that prostaglandin (PG) and cyclooxygenase (COX) plays an essential action in the inflammatory progression through changing the synthesis of prostaglandins from arachidonic acid. There are two isoforms of cyclooxygenase namely COX-1 and COX-2 (Gao et al., 2014) which are differentially expressed. COX1 is expressed in most tissues and is responsible for the biosynthesis of PGs and regulation of renal and - platelet functions. Whereas, COX-2 can be quickly induced by inflammatory stimuli and a significant number of mutagenic and pro-inflammatory cytokines that lead to increased levels of prostaglandins which can have an effect on cell proliferation,

apoptosis, and contributes to cancer transformation (Kuroda and Yamashita, 2002). Satyendra Singh Baghel et al., (2012) pointed out that the G1-M phase of the cell cycle checkpoint was controlled by the p53 gene which plays a significant part in cell proliferation (Baghel et al., 2012). The p53 gene is classified as a tumour suppressor (Kim et al., 2015; Bieging et al., 2014). It has been illustrated that the decrease of p53 expression, especially in the ageing, allows the cancer transformation (Kim et al., 2015). Gibellini et al., (2010) demonstrated that p53 could trigger apoptosis and control programmed cell death; moreover, quercetin can initiate the apoptosis in cancer cells through the upregulation of Bak and Bax and the downregulation of Bcl-2 (Gibellini et al., 2010). The Bcl-2 is the best indicator for protein families, which contributes to the control of programmed cell death, by regulating pro-apoptotic and anti-apoptotic factors. The Bcl-2 and Bcl-XL are anti-apoptotic factors that can regulate apoptosis either by sequestering caspases or by blocking the production of mitochondrial apoptogenic factors such as cytochrome c and apoptosis-inducing factor (AIF). Once inside the cytoplasm, cytochrome c and AIF immediately stimulate caspases that attach to cellular proteins leading to apoptotic changes. Bax and Bak trigger the producing of caspases from death antagonists via inducing the production of mitochondrial apoptogenic factors or mitochondrial permeability transition pore, that way leading to caspase activation (Tsujimoto, 1998). Quercetin is considered as the inducer of apoptosis in cancer and inflammation via Bax, Bak and Bcl-2. Once the cells have been treated by quercetin, the quercetin induces the upregulation of Bax and Bak, and downregulation of Bcl2 that guides the cell to apoptosis (Gibellini et al., 2010).

This study aims to examine the effect of both bulk and nano forms of quercetin on the expression of COX1, COX2, and p53 and Bcl-2 genes in lymphocytes collected

from patients with TB and compare them with those from healthy donors using the qPCR technique. The rationale for selecting the QPCR technique is that it is robust and offers a high level of specificity and sensitivity compared with other techniques such as western blot analysis

6.3 Material and Methods

The material and methods as well as experimental design were listed and described in Chapter 2 Material and Methods section (2.13, 2.13.1, 2.13.2) page number 62-65.

6.4 Results

6.4.1 Both forms of quercetin reduce expression of COX1 mRNA in lymphocytes of healthy individuals and patients with TB.

The expression of COX1 transcripts was investigated in lymphocytes collected from healthy individuals and patients with TB. 140μM of PhIP induced optimal DNA damage in lymphocyte as shown in figures 59 and 60. Lymphocytes were treated by 140μM of PhIP and corresponding lymphocytes were measured in the presence of either bulk or nano forms of quercetin incubated for 2 hours. The gene expressions were normalised against a housekeeping gene (β actin) using the ΔΔCq. The quercetin nano form showed significant downregulation in the COX1 gene compared with untreated cells and 140μM of PhIP in healthy individuals (Figure 69 and 70). The P value was **$p \leq 0.0074$ with the One-Way ANOVA test. The down-regulation of the COX1 gene was observed in lymphocyte from TB patients treated with both bulk and nano forms but was significant only in lymphocytes treated with the nano form of quercetin when compared with the untreated and PhIP induced cells.

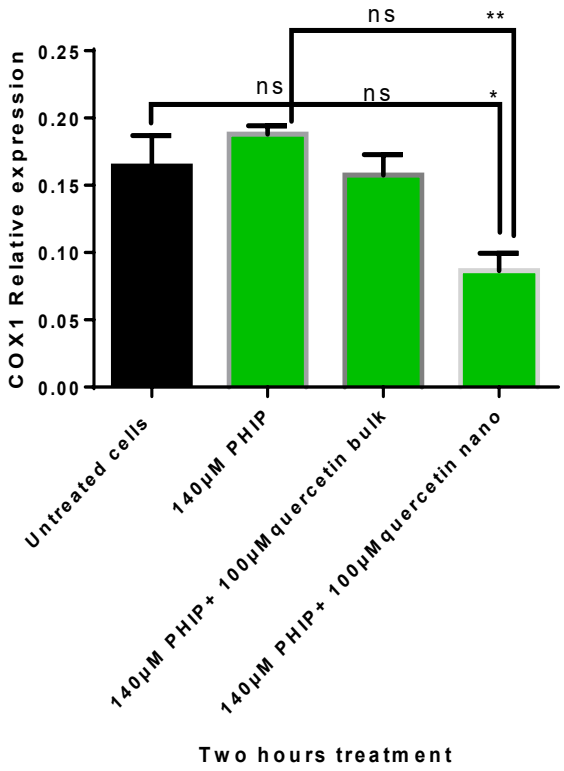


Figure 69: The effect of both forms of quercetin using Real-time PCR (qPCR), data expressed in relative changes of COX1 mRNA over the control, using β actin as an endogenous housekeeping gene in lymphocytes collected from three different healthy individuals. The Relative expression level of COX1 mRNA after treatment with bulk and nano forms of quercetin. Results are expressed as mean ± SEM, One-Way AVOVA test and significance compared with the untreated cells and 140µM of PhIP (donors =3).

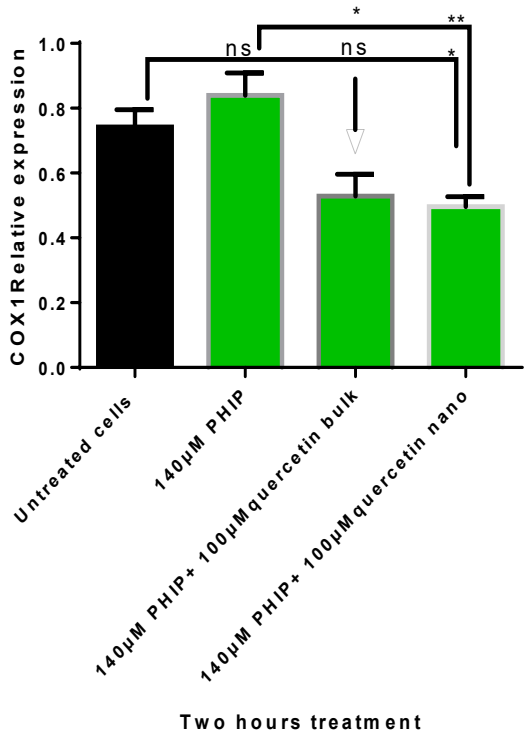


Figure 70: The effect of both forms of quercetin using Real-time PCR (qPCR), data expressed in relative changes of COX1 mRNA over the control, using β actin as an endogenous housekeeping gene in lymphocytes collected from three different TB patients. The Relative expression level of COX1 mRNA after treatment with bulk and nano forms of quercetin. Results are expressed as mean ± SEM, One-Way AVOVA test and significance compared with the untreated cells and 140µM of PhIP (donors =3).

6.4.2 The bulk and nano forms of quercetin had no significant effect on the expression of COX2 mRNA in lymphocytes collected from healthy individuals and TB patients treated with 140μM of PhIP.

The COX2 gene expression was investigated using our isolated lymphocytes collected from healthy individuals and TB patients. 140μM of PhIP induced optimal DNA damage in lymphocyte as shown in figures 59 and 60. Lymphocytes were treated by 140μM of PhIP and corresponding lymphocytes were measured in the presence of either bulk or nano form of quercetin incubated for 2 hours. The 140μM of PhIP does not induce COX2 down-regulation in lymphocytes that treated with 100μM with both bulk and nano forms of quercetin. The experiments were designed in triplicate, and all the expressions were normalised against a housekeeping gene (β actin). The ΔCq was used to represent statistical analysis. The down-regulation was compared to untreated cells and 140μM of PhIP (figures 71 and 72). Both forms of quercetin, 140μM of PhIP and untreated cells were normally distributed.

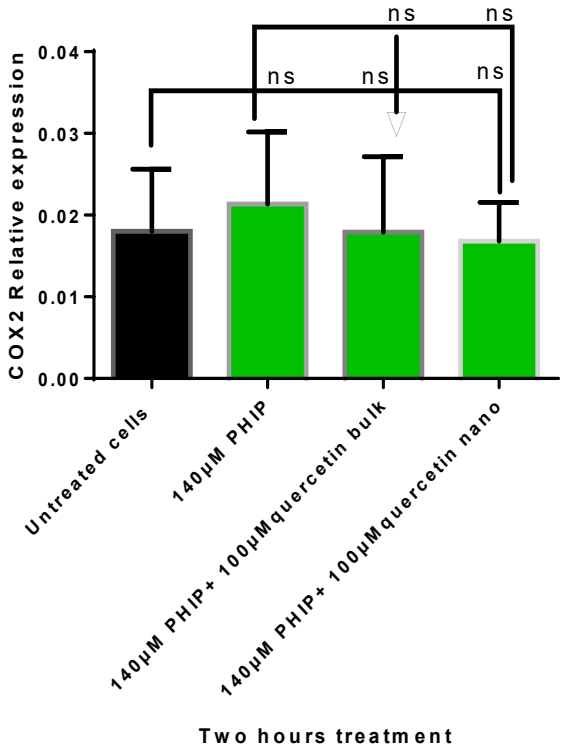


Figure 71:The effect of both forms of quercetin using Real-time PCR (qPCR), data expressed in relative expression of COX2 mRNA over the control, using β actin as an endogenous housekeeping gene in lymphocytes collected from three different healthy individuals. The Relative expression level of COX2 mRNA after treatment with bulk and nano forms of quercetin. Results are expressed as mean ± SEM, One-Way AVOVA test and significance compared with the untreated cells, One-Way AVOVA teste cells and 140µM of PhIP (donors=3).

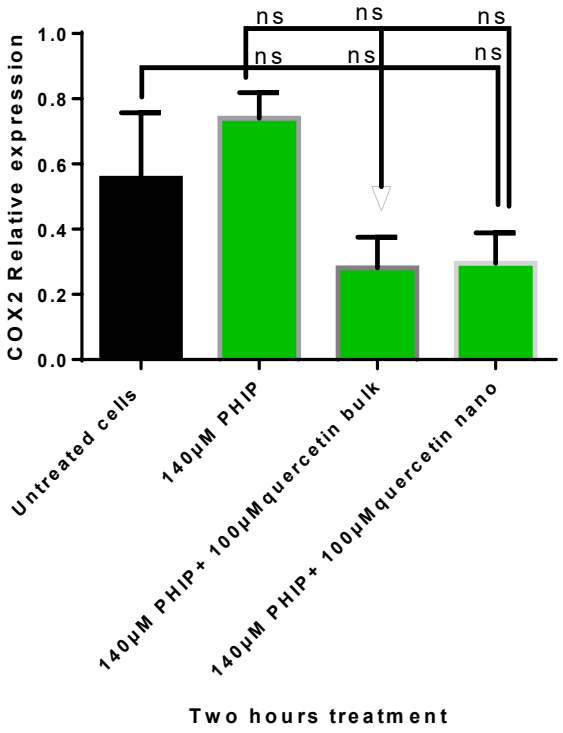


Figure 72: The effect of both forms of quercetin using Real-time PCR (qPCR), data expressed in relative expression of COX2 mRNA over the control, using β actin as an endogenous housekeeping gene in lymphocytes collected from three different TB patients. The Relative expression level of COX2 mRNA after treatment with bulk and nano forms of quercetin. Results are expressed as mean ± SEM, One-Way AVOVA test and significance compared with the untreated cells and 140µM of PhIP (donors=3).

6.4.3 P53 mRNA expression was upregulated but not significantly in lymphocytes collected from healthy individuals and TB patients treated with both forms of quercetin in the presence of 140μM of PhIP.

The figures 73 and 74 provide information about P53 gene expression in lymphocytes collected from healthy individuals and patients with TB. 140μM of PhIP induced optimal DNA damage in lymphocyte as shown in figures 59 and 60. Lymphocytes were treated by 140μM of PhIP and corresponding lymphocytes were measured in the presence of either bulk or nano form of quercetin that incubated for 2 hours. The 140μM of PhIP did not induced p53 upregulation, and the changes were quantified using quantitative PCR. Untreated cells and cells treated with140μM of PhIP only were used for comparison to determine the value of the p53 expression. The experiments were designed in triplicate, all expressions were normalised against β actin using the ΔCq livak method. The results showed that there is non-significant up-regulation in p53 expression after two hours of treatment in both lymphocytes from healthy individuals and patients with TB treated with the bulk form of quercetin (figures 73 and 74).

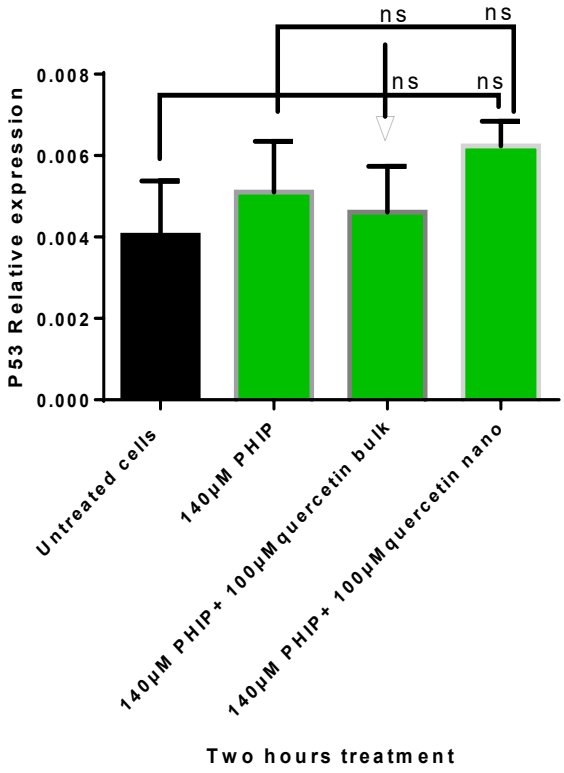


Figure 73: The effect of both forms of quercetin using Real-time PCR (qPCR), data expressed in relative expression of p53 mRNA over the control, using β actin as an endogenous housekeeping gene in lymphocytes collected from three different healthy individuals. The Relative expression level of p53 mRNA after treatment with bulk and nano forms of quercetin. Results are expressed as mean ± SEM, One-Way AVOVA test and significance compared with the untreated cells and 140µM of PhIP (donors =3).

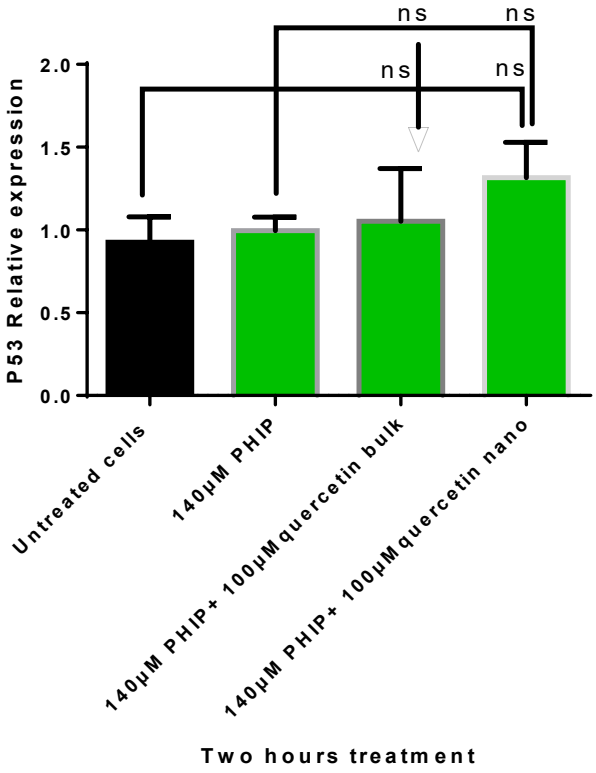


Figure 74: The effect of both forms of quercetin using Real-time PCR (qPCR), data expressed in relative expression of p53 mRNA over the control, using β actin as an endogenous housekeeping gene in lymphocytes collected from three different TB patients. The Relative expression level of p53mRNA after treatment with bulk and nano forms of quercetin. Results are expressed as mean ± SEM, One-Way AVOVA test and significance compared with the untreated cells and 140µM of PhIP(donors=3).

6.4.4 Bcl-2 mRNA expressions was significantly down regulated in lymphocytes from TB patients treated with the nano form of quercetin in the presence of 140μM of PhIP.

Bcl-2 gene expression changes in lymphocyte collected from healthy individuals and patients with TB treated with both forms of quercetin 100μM was specified using quantitative PCR. 140μM of PhIP induced optimal DNA damage in lymphocyte as shown in figures 59 and 60. Lymphocytes were treated by 140μM of PhIP and corresponding lymphocytes were measured in the presence of either bulk or nano form of quercetin, two hours incubation was used to assess gene expression. All the gene expressions were normalised against β actin using the ΔCq livak method, the experiments were designed in triplicate. Figure 75 showed that there was no significant (ns) change in Bcl-2 expression in treatments with bulk and nano forms compared to untreated cells, whereas the comparison with 140μM of PhIP illustrated significant down-regulation where it was *p ≤ 0.0401. The lymphocytes from TB patients demonstrated a non-significant change in Bcl-2 expression by bulk treatment compared to untreated cells. The non-significance difference was seen for two hours incubation using bulk form, while the expression of Bcl-2 was influenced statistically by the presence of the quercetin nanoparticle, the effect showed a steady, significant decrease **p ≤ 0.0060 at two hours incubations compared with untreated cells and 140μM of PhIP fiqure76.

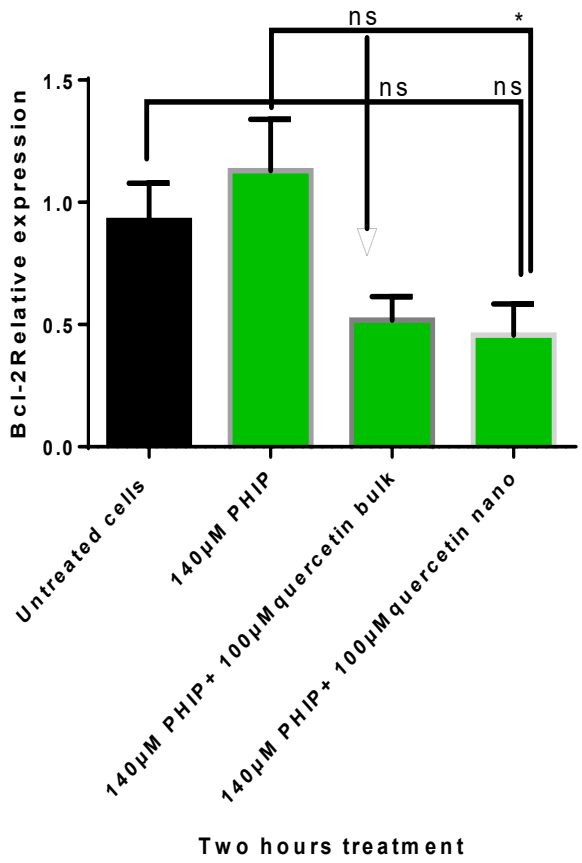


Figure 75: The effect of both forms of quercetin using Real-time PCR (qPCR), data expressed in relative expression of Bcl-2 mRNA over the control, using β actin as an endogenous housekeeping gene in lymphocytes collected from healthy individuals. The Relative expression level of Bcl-2 mRNA after treatment with bulk and nano forms of quercetin. Results are expressed as mean ± SEM, One-Way AVOVA test and significance compared with the untreated cells and 140µM/ml of PhIP (donors=3).

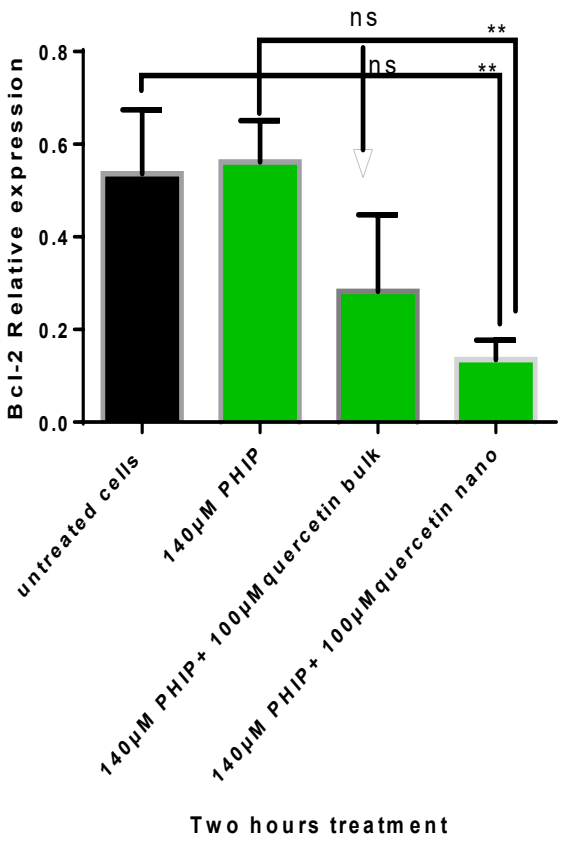


Figure 76: The effect of both forms of quercetin using Real-time PCR (qPCR), data expressed in relative expression of Bcl-2 mRNA over the control, using β actin as an endogenous housekeeping gene in lymphocytes collected from TB patients. The Relative expression level of Bcl-2 mRNA after treatment with bulk and nano forms of quercetin. Results are expressed as mean ± SEM, One-Way AVOVA test and significance compared with the untreated cells and 140µM/ml of PhIP (donors=3).

6.5 Discussion

Many studies have investigated the correlation between quercetin and cyclooxygenase COX-1 and COX2, but the results are inconsistent. Studies suggested that regular intake of quercetin is protective against the inflammatory process and cancer (Wach et al., 2007; Russo et al., 2012). COX1 can originate in any location of inflammation, while COX2 can be directly activated by an inflammatory stimulus and produces a large amount of pro-inflammatory mitogens and cytokines that increase the proportion of prostaglandins and affect cell proliferation that finally leads to malignant transformations (Kuroda and Yamashita, 2002).

The effect of quercetin both bulk and nano forms on the expression of the COX1, COX2, P53 and Bcl-2 were investigated in lymphocytes from three different patient with TB and three different healthy individuals. The data illustrated that quercetin had no significant effect on COX2 expression in lymphocytes from healthy individuals and TB patients' figures (71, 72). The expression of the COX1 gene was significantly downregulated in lymphocyte from healthy individuals and TB patients when treated with quercetin. Quercetin bulk decreased the expression of COX1 significantly in the lymphocytes from TB patients compared to 140μM of PhIP *P≤0.0120, whereas the comparison with untreated cells showed a non-significant effect. In samples treated with the nano form of quercetin, the statistical differences showed significant changes with both lymphocytes from healthy individuals and TB patients compared to 140μM of PhIP where the P value showed *P≤0.0120 and **P≤0.0074 figures (69 and 70). Affinity results were suggested by Ribeiro (2014), that flavonoid down-regulate COX-1 and COX-2 in human whole blood, also reported that all flavonoids have an arylbenzopyran ring system, and different kinds of flavonoids originated

from different rearrangements (Ribeiro et al., 2014). Moreover, Banerjee (2002) pointed out that quercetin, at 50 μM concentration downregulate the COX-2 mRNA and protein expression (Banerjee, Van der Vliet and Ziboh, 2002).

Moon et al., (2003) reported that quercetin has inhibitory effects on vascular smooth muscle cells. They found that quercetin treatment induced a cell-cycle arrest in G1-phase, also it down-regulated of cyclins and CDKs and up-regulated of the CDK inhibitor p21 expression, whereas up-regulation of p53 by quercetin was not witnessed. Our study agrees with the findings of Moon et al, and showed that the two forms of quercetin had a non-significant impact on p53 expression in lymphocytes collected from both healthy individuals and TB patients. The data displayed non-significant up-regulation of p53 in lymphocytes collected from TB patients ($P \leq 0.05$). In a combination of the quercetin with irradiation to treat the ovarian cancer cells, the results showed that upregulation of Bax level and downregulation of Bcl-2 level in OV2008 and SKOV3 cell lines compared with that in the cells exposed to quercetin or X-rays alone. Furthermore, knocking down of p53 by particular siRNA considerably reduced on increase of DSBs (Gong et al., 2017). Maurya and Vinayak, (2015) concluded that quercetin produced anticarcinogenic action by upregulation of p53 and BAX in HepG2 cells through downregulation of PKC, ROS, COX-2, and PI3K.

Our data also demonstrated that the quercetin nano form was more effective in down regulation of COX1 expression; moreover, the quercetin bulk form had no significant effect on Bcl-2 expression in lymphocytes collected from healthy individuals and TB patients, while the nano form of quercetin showed significance in lymphocytes from both healthy individuals and TB patients. Subsequently, we found that quercetin had a statistically significant effect on the change in Bcl-2 expression in lymphocytes

from healthy and lymphocytes from patient with TB where the p value ranged from *p ≤ 0.00401 to **p ≤ 0.0060 respectively. The significant result as the down regulation of Bcl-2 could adversely affect the survival of the M*ycobacterium* as over expression of Bcl-2 mRNA is a mechanism that leads to anti-apoptotic activity in macrophage cell lines ensuring the survival of the *mycobacteria* within these cells (Zhang et al., 2008). This suggests a possible role for quercetin, especially the nano form, in genoprotection influence to TB through its enzyme modulating effect.

7 Chapter (7) Investigation of catalase proteins in lymphocytes collected from healthy individuals and Patients with TB treated with quercetin using the Western Blot technique

7.1 Investigation of effect of quercetin both forms on catalase protein production in lymphocytes from healthy individuals and patients with TB using Western blot technique.

7.2 Introduction

Flavonoids have long been linked with a wide range of health promoting effects due to their anti-oxidative, anti-inflammatory, anti-mutagenic, anti-carcinogenic and enzyme modulating properties. Regular intake of flavonoids can have a protective effect against a number of diseases, including lung cancer (Serafini et al. 2010). Quercetin can activate catalase which is a common enzyme found in nearly all living organisms and very important in protecting the cells from oxidative damage caused by ROS. It also catalyses the breakdown of hydrogen peroxide to water and oxygen, where one molecule of this enzyme can transform millions of hydrogen peroxide molecules to water and oxygen in less than one minute (Dieterich et al., 2000). Habas et al., (2018) stated that the effect of the IQ food mutagen on the catalase as an endogenous antioxidant enzyme was studied using lymphocytes collected from COPD patients, the hypothesis included DNA damage due to the production of oxidative stress caused by ROS. The data shows that catalase mRNA expression levels are significantly increased, and catalase action may play an important role in reducing DNA damage in the lymphocytes of COPD patients. However, the cell signalling molecules regulate catalase production to reduce DNA damage through PKA and PKCζ activators of forskolin and okadaic acid increased catalase activity (Yano and Yano, 2018). Moreover, catalase expression has been linked with numerous diseases. For instance, certain polymorphisms in the catalase gene have been detected in Alzheimer disease, hypertension, and diabetes. Reduced catalase activity has been described as acatalasemia (Glorieux et al., 2015). Nishikawa et al, (2009) pointed out that the CAT gene expression can control the levels of catalase;

the gene expression is detected at the transcription, post-transcription and post-translation levels (Nishikawa et al., 2009). It has been shown that the CAT gene expression can be affected by the peroxisome proliferator activated receptor γ (PPARγ). PPARγ is a ligand-activated transcription factor that might control the expression of the CAT gene by binding to a distal PPARγ response element (PPRE) in its promoter region (Kodydkov á J et al., 2014). Gargouri et al, (2011) studied the protective influence of quercetin versus oxidative stress produced by dimethoate in human lymphocytes using a spectrophotometer to measure the enzyme levels; they reported that the breakdown of H_2O_2 to nontoxic compounds is the major function of catalase and the enzyme activities such as, CAT and SOD were significantly increased and linked with antioxidant defence mechanisms. Studies on lymphocytes from colorectal cancer patients and healthy donors using the Comet assay showed that the DNA damage induced by PhIP in lymphocytes was effectively decreased by supplementation with the quercetin as an ant-oxidant (Najafzadeh et al., 2009).

In the present study, we investigated the expression of mRNA of catalase protein in lymphocytes collected from healthy individuals and patients with TB. PhIP (140μM) was used as a DNA damage inducer; the cells were treated with both forms (bulk and nano) of quercetin individually.

The Western blot method was used in this project, to study the effect of two quercetin forms on the expression of mRNA catalase protein as it a commonly used research tool to detect and evaluate proteins. In this technique, different types of proteins are isolated on the basis of their molecular weight, through gel electrophoresis. The segregated proteins are then transferred on to a special

membrane called PVDF/nitrocellulose and incubated with specific primary and secondary antibodies (Mahmood and Yang, 2012). However, the specific antibodies are attached with target proteins forming protein bands on the membrane, the bands can be easily imaged using (ECL) mixture and an imaging box. The size of the bands reflects the amount of proteins; consequently, a standard can be used to detect the amount of protein found at a certain level expressed in Kilo Daltons (kDa) (Mahmood and Yang, 2012).

7.3 Material and methods

All the material and method used in the Western blot technique were mentioned and displayed in chapter 2 section (2.14, 2.14.1, 2.14.2, 2.14.3, 2.14.4, 2.14.5, 2.14.6 and 2.14.7) page number 66-70.

7.4 Results

7.4.1 Catalase expression in lymphocytes from healthy individuals treated with bulk and nano forms of quercetin in the presence of PhIP (140μM).

Figure 77 shows the catalase protein expression using isolated lymphocytes collected from healthy individuals. 140μM of PhIP induced optimal DNA damage in lymphocyte as shown in figures 59 and 60. Lymphocytes were treated with 140μM of PhIP and corresponding lymphocytes were measured in the presence of either 100 μM bulk or nano forms of quercetin. PhIP (140μM) does not modulate the expression of catalase in lymphocytes. Two hours incubation time was used for all treatments. Untreated cells were used as a control to detect the level of catalase expression for comparison at 60 KDa. Incubation with PhIP alone or in the presence of bulk or nano forms of quercetin had no effect on catalase protein expression.

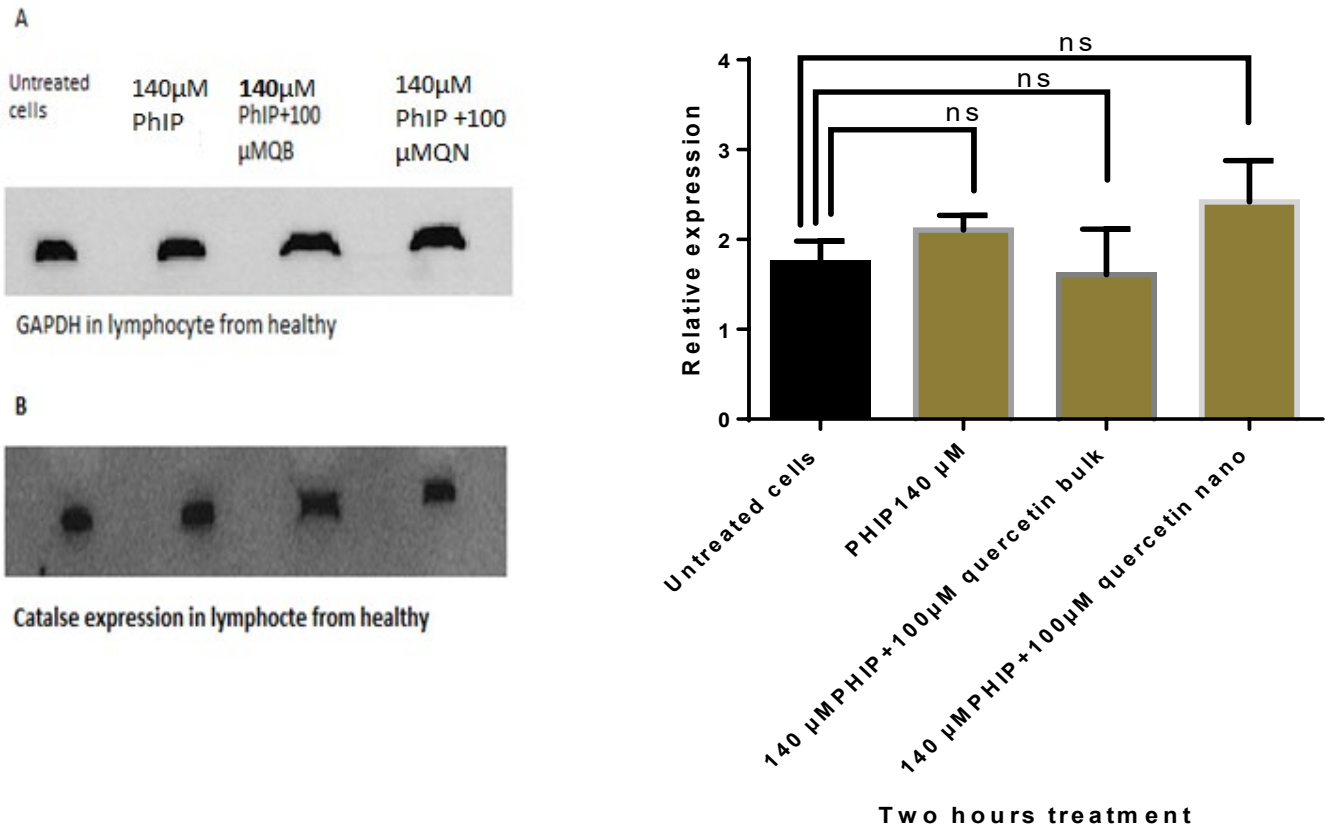


Figure 77: The effect of quercetin both forms on catalase protein expression in lymphocytes collected from three different healthy individuals incubated for 2 hours and (A, B) developed bands of catalase, and GAPDH by immunoblotting at 60 kDa. GAPDH was used as a control, ±SEM, One-Way AVOVA test and significance compared to untreated cells, 140µM of PhIP (donors =3).

7.4.2 Catalase expression in lymphocytes from patient with TB treated with bulk and nano forms of quercetin in the presence of PhIP (140μM).

The figure 78 illustrates the effect of quercetin on catalase protein expression in isolated lymphocytes from TB patients where DNA damage was induced by PhIP. 140μM of PhIP induced optimal DNA damage in lymphocyte as shown in figures 59 and 60. Lymphocytes were treated with 140μM of PhIP for two hours and corresponding lymphocytes were assessed for changes in the expression of catalase in the presence of either 100 μM bulk or nano forms of quercetin. Untreated cells were considered as a control to detect the quantity of catalase expression for comparison. A significant up-regulation for catalase protein was observed in lymphocytes from patients with TB treated with nano form of quercetin $*p \leq 0.0236$, the up-regulation of catalase can be noticed at 60 KDa (Figure 78). On the other hand, up-regulation of the catalase in lymphocytes from TB patients was non-significant, using the bulk form of quercetin. Both forms of quercetin, untreated cells and 140μM of PhIP were normal distributed.

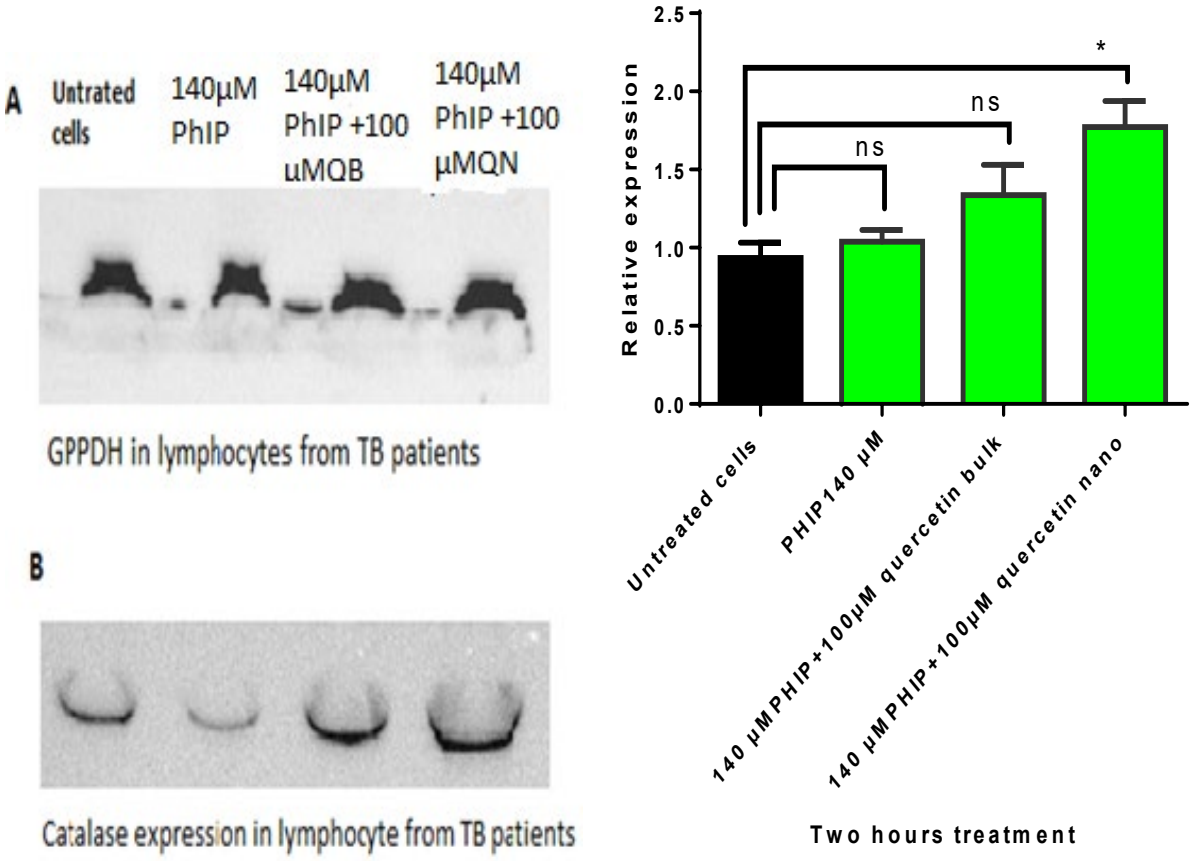


Figure 78: The effect of quercetin both forms on catalase protein expression in lymphocytes from three different patients with TB incubated for 2 hours and (A, B) developed bands of catalase, and GAPDH by immunoblotting at 60 KDa. GAPDH was used as a control. ±SEM, One-Way AVOVA test, and significance compared to untreated cells and 140µM of PhIP (donors =3).

7.4.3 Comparison of Catalase protein expression in PhIP induced DNA damaged lymphocytes from healthy individuals and patients with TB, treated with bulk and nano forms of quercetin.

The comparative effects of quercetin bulk and nano forms on catalase protein expression using lymphocyte from healthy individuals and TB patients are shown in the bar chart 79, the expression of catalase protein displayed a non-significant (ns) change when treated by the bulk form of quercetin, using lymphocytes from healthy individuals and TB patients. The effect of quercetin nano was non-significant $p \geq 0.05$ in protein expression in lymphocyte from healthy individuals. By contrast, the quercetin nano was the only treatment which showed significant up-regulation in lymphocytes from patients with TB *$p \leq 0.0236$ and represented the only significant upregulations. The GPPDH was used as a control demonstrating relative expression of catalase after treatment with quercetin both forms individually.

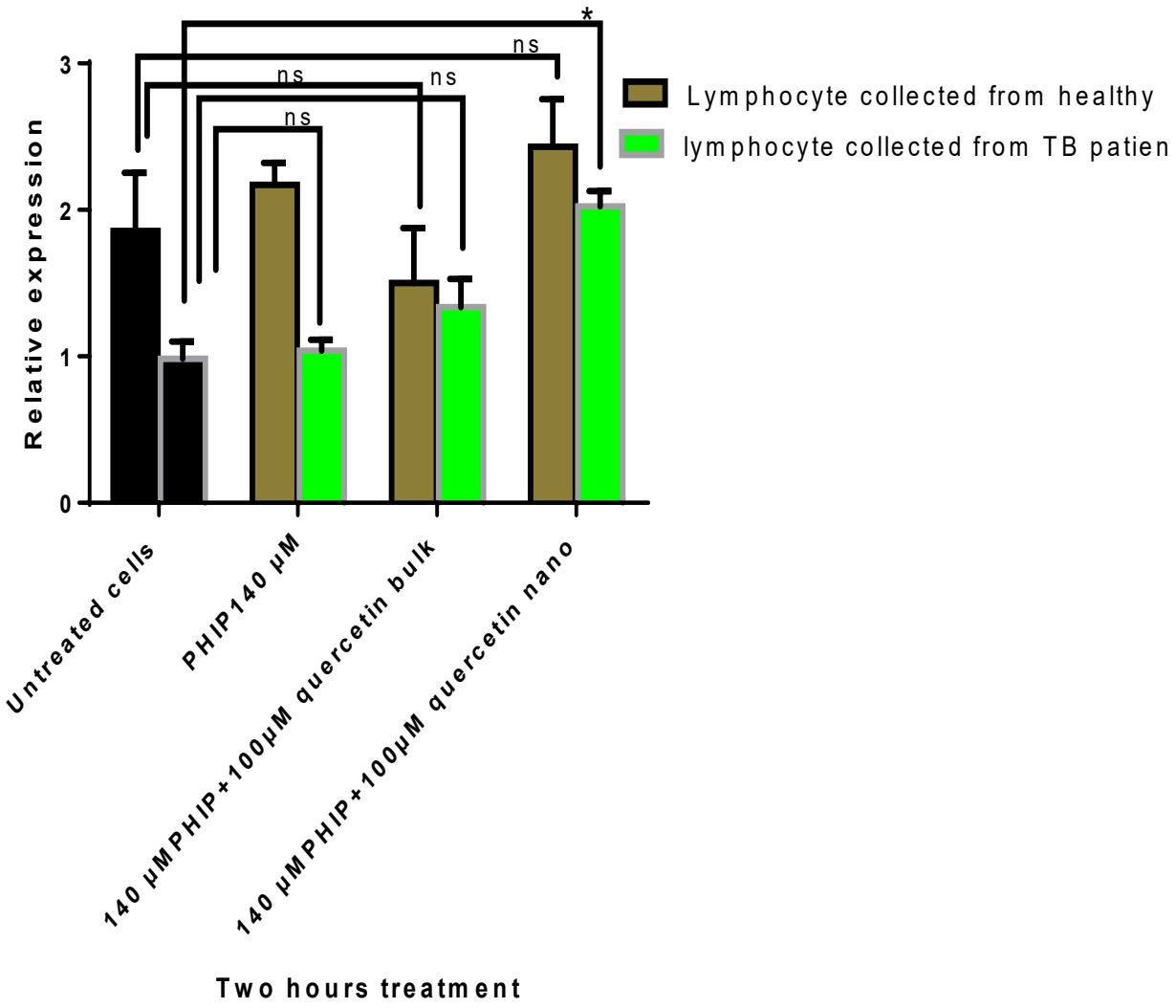


Figure 79: A comparison of the effect of quercetin in both nano and bulk forms on catalase protein expression in lymphocytes from three different healthy individuals and three different TB patients incubated for 2 hours and GAPDH by immunoblotting at 60 kDa. GAPDH was used as a control. ±SE, Two-Way ANOVA test and significance compared to untreated cells and 140µM of PhIP (donors =3).

7.5 Discussion

The DNA comes in contact with different damaging compounds daily. In normal cells the repair mechanism can protect from DNA damage; if the repair mechanism is dysfunctional it could lead to DNA disorder which could accumulate and lead to mutations. Some of these mutations could lead to development of cancer (Chakarov et al., 2013). The food mutagen, PhIP and IQ, are heterocyclic amines (HCA), produced while heating proteinaceous food, and could be a reason for oxidative stress that finally causes DNA damage (Kurzawa-Zegota et al., 2012). Investigations on the protective effect of quercetin against oxidative stress induced by IQ and PhIP in *vitro* using lymphocytes from healthy individuals and colon cancer patients showed that the DNA damage caused by a high dose of IQ and PhIP was significantly ***p≤ 0.001 reduced when treated by quercetin (Kurzawa-Zegota et al., 2011). The current study uses the Western blotting technique to examine the potential genoprotective effect of nano- and bulk- forms of quercetin 100 μM; the effect was examined through enzyme modulating properties of the catalase as an anti-oxidant.

For catalase protein expression, lymphocyte from healthy individuals treated with the anti-oxidant, incubated for two hours after treatment had a non-significant increase in its expression for both quercetin bulk and nano P≤ 0.05. However, for both treatments (nano and bulk) there was a change in catalase protein expression compared to the untreated cells. Habas et al, (2018) studied that the detection of catalase high-expression by ROS was changeable. Cells were treated with the food mutagen IQ 140μM and the levels of catalase mRNA was detected in lymphocytes from COPD patients. The results illustrated that IQ

significantly increased mRNA expression of catalase compared with untreated cells. They concluded that the anti-oxidant compound such as quercetin has the ability to reduce oxidative stress and stimulated protective enzyme systems, in lymphocytes from healthy individuals and COPD patients. Therefore, quercetin could be very beneficial in decreasing oxidative DNA damage in lymphocytes from COPD patients and consequently decrease the risk of possible cancer and other respiratory disease (Habas et al., 2018). In the current study lymphocytes collected from TB patients, the catalase protein expression was more productive with the quercetin nano form and was statistically significant *P ≤ 0.0236 compared to the untreated cells. There is non-significant catalase protein production with quercetin bulk form, because this expression was rated at p ≤ 0.05 using lymphocytes from TB patients. This is in line with many studies that report an important role for endogenous catalase, and catalase mRNA in stressful conditions. For instance, catalase is upregulated in the human heart after concern of a stress suffering. Dieterich et al, (2000) pointed out that amplified oxidative stress in transmural tissue samples collected from the left ventricle after human heart failure leads to upregulation of catalase gene expression as a compensatory mechanism, whereas glutathione peroxidase (GPX) and superoxide dismutase (SOD) gene expression were normal. Similarly, the oxidative stress and apoptotic factories relationship have been investigated in lymphocytes from diabetic patients using the Western blot technique, and it has been found that catalase activity is decreased in both groups healthy and patients compared to untreated cells. This decrease in CAT activity could result from inactivation by glycosylation of the enzyme, or by the accumulation of superoxide radicals and hydrogen peroxides (Arya et al.,

2011). Results from Western blot technique indicate that the nano form of quercetin was more effective than the bulk form, in the lymphocytes from TB patients, with an upregulation of the catalase protein *$p \leq 0.0236$ and had a higher genoprotective effect suggesting a possible role for quercetin, especially the nano form, in genoprotection through its enzyme modulating effect among patients with TB.

8 Chapter (8) General Disscusion

8.1 General Discussion

Genotoxicity can be defined as any alteration or damage by a toxicant in the genome, which under some conditions correlates to mutagenicity. Hence, genotoxicity can be divided into different gene alterations (insertion, deletion and point mutation), clastogenic impacts (breakage in DNA strands leading to alterations in chromosomes structures), and aneugenic defects (numeral chromosomal aberrations leading to the development of aneuploidy or polyploidy) (Eastmond et al., 2009). Genetic mutation and DNA damage can be introduced by different factors having genotoxic potential such as chemical substances and physical agents (UV and X radiation). This genetic alteration could be detrimental where it increases the chance of cancer, congenital defects, and inflammation. Consequently, genotoxicology research becomes an essential part of biomedical science that enable scientists to study and discover the impact of other chemicals, and physical agents. Lymphocytes can be suitable reflect cells for cancer and other diseases to determine DNA damage caused by a chemical or physical substance that contains exogenous or endogenous genotoxins (Najafzadeh et al., 2012). Hence, lymphocytes have been selected for this study to examine DNA damage.

Tuberculosis is a disease caused by *Mycobacterium tuberculosis* which affects around 10 million people worldwide. Though the incidence of TB in England has shown a decline in the past 5 years, the globalisation and the fast and easy travel opportunities has created a situation where TB can be swiftly exported anywhere in the world (Global tuberculosis report (2017) (Glorieuxet al.,2018). Moreover, the TB mortality is higher in urban areas particularly London and the

emergence of multidrug resistant TB has made the control of TB more complicated. Hence it is important to identify compounds that supplement the current TB treatment options.

Several studies have suggested that the regular intake of flavonoids, such as quercetin, lead to a wide range of positive health effects due to their anti-oxidative, anti-inflammatory, anti-mutagenic, anti-carcinogenic, and enzyme modulating properties; furthermore, the regular intake of flavonoids has also been shown to have a protective effect against a number of diseases, including lung cancer (Johnson and Loo, 2000; Nijveldt et al., 2001). The protective effect of quercetin is thought to be mediated by the regulation of different pathways, including the inhibition of COX1 and COX2, and the up-regulation of certain tumour suppressor genes, such as p53 and Bax and Bak. This can lead to the down-regulation of Bcl-2 and Bcl-xL, which help guide cells into apoptosis (Gibellini et al., 2010). A survey of literature has identified a gap in investigations on the beneficial effect of different (bulk and nano) forms of quercetin among TB patients. Consequently, in this study, quercetin nanoparticles and bulk forms were examined for their impact on DNA damage using lymphocytes from both healthy individuals and patients with TB. Nanotechnology refers to the application of materials at a scale of one-billionth of a meter. Once the materials are reduced to this nano form, they display newly improved properties, including physicochemical and biological characteristics, compared to those of bulk materials. Moreover, they can penetrate cell membranes in several ways, particularly in mitosis division wherein the nanoparticles (NPs) become enclosed in the nucleus membrane,

allowing for direct contact with the DNA due to the disintegration of the nucleuar membrane (Ochekpe et al., 2009, Singh et al., 2009).

This study began with the collection of 20 blood samples and 20 sperm samples from different healthy individuals. The control samples were examined with different concentrations of hydrogen peroxide to detect which concentration causes the highest DNA damage (Figures 14 and 15). The concentration 60μM of hydrogen peroxide was chosen as a positive control for the lymphocytes. Additionally, the blood was treated with bleomycin in the micronucleus assay to detect the maximum DNA damage (Figure 44 and table 9). The concentration of 0.75μg/ml was identified as causing the highest levels of damage in the micronucleus assay. 50μl of MITC (0.4μM) was used as a positive control in the micronucleus assay, acting as a system control, whereas the negative control was medium and original excipient.

Moreover, the concentrations 160μM of IQ and 140μM of PhIP food mutagen were selected as the genotoxic doses causing the highest DNA damage without toxicity in lymphocytes from healthy individuals, these doses were used as a DNA damage inducer (Henderson et al., 2000) (Figures 49, 50, 59, and 60). A concentration of 50μM hydrogen peroxide was used as a positive control for the sperm experiment (Figures 16 and 17), and a concentration of 100μM and 120 μM of food mutagens IQ and PhIP were identified as that can cause the highest DNA damage in sperm from healthy individuals (Figures 55-56, 65-66).

The project results were evaluated when quercetin was combined with hydrogen peroxide in the Comet assay, with bleomycin in the micronucleus assay, and with PhIP in qPCR and Western blot techniques. The differences

between the bulk and nano forms of quercetin in each assay are shown in (Figures 38, 39 and 46). The effect of quercetin on both cells' types, haploid (germ cells) and diploid (lymphocytes) are illustrated in (Figures 30, 31, 32, and 33).

8.2 The Effect of the Bulk and Nano Forms of Quercetin on DNA Damage via the Comet Assay

The comet assay can be defined as a micro-electrophoretic assay that allows for the direct measurement and imaging of DNA damage (Ostling & Johanson, 1984). This test has become the globally preferred assay due to its simplicity: small amount of blood samples is needed, making it inexpensive compared to other techniques (Anderson et al., 1991). In 2000, Tice et al. reported that the percentage of head and tail DNA damage could be efficiently investigated by the Comet assay wherever the head displays undamaged DNA, whereas the tail can identify damage (Tice et al., 2000). In this study both forms of quercetin (bulk and nano) were separately incubated in vitro at 37°C for 30 min. Subsequently, the lymphocytes were electrophoresed in alkaline conditions P^H ≥13, which was followed by staining and scoring. Three different concentrations, 10μM, 25μM, and 100μM, of quercetin, bulk and nano, were used. Using Statistical Package for the Social Sciences (SPSS) and graph pad prism 6, a One-Way ANOVA test showed a non-significant effect of the lymphocytes collected from healthy individuals treated with the bulk form; these results were compared to the positive control of 60 μM of hydrogen peroxide and exhibited a P value P ≥ 0.05 (Figures 18 and 19). The same concentrations were used to treat the lymphocytes collected from the TB patients; however, in this case, the nano form was observed to be significant at 25 μM with *P ≤ 0.0283 and 100

μM *** P ≤ 0.001. The bulk form was non-significant in treating the same cells (Figures 24 and 25), and this may refer to cellular metabolism process in lymphocytes collected from healthy individuals can stop chemically-induced DNA damage by either DNA repair or apoptosis where the machinery repair not working efficiently in inflammatory or cancer cells (brendler et al.,2005)

The DNA damage levels in spermatozoa collected from healthy individuals were higher than that in the lymphocytes of the healthy individuals (Figures 30 and 31). Previous researchers have pointed out that spermatogenic cells exhibit a higher mutation frequency than somatic cells (Hess 2005, Walter et al. 1998; Winn et al. 2000). These results confirmed that sperm are more sensitive for DNA damage than lymphocytes, since sperm consists of haploid cells, while lymphocytes consist of diploid cells. Moreover, there was a substantial reduction in DNA damage when the spermatozoa were treated with 25 μM and 100 μM of quercetin nano form. Statistically, the One-Way ANOVA test presented *P≤0.0283 with 25μM and ***P≤0.001 with 100 μM (Figures 32 and 33). In regard to the bulk form that treated spermatozoa in the presence of 50 μM of H_2O_2, the reduction was not observed to be significant, with the value P ≥0.05 (Figure 30 and 31). These results were in accordance with the fact that reducing the size to the nano form can increase the surface area, solubility, immunogenicity, and drug delivery. Furthermore, NPs have a greater ability to penetrate the nuclear membrane than the bulk size (Zhang et al., 2008, Singh et al., 2009).

8.3 The Effect of the Bulk and Nano Forms of Quercetin on MNi Lymphocytes via the Micronucleus Assay

A micronucleus is a smaller, isolated, extra fragment produced when a chromosome fails to combine with the spindle fibres during the mitosis stage. Additionally, centric components or a full chromosome can form the MNi. This abnormal structure reflects cytogenetics damage. This assay was selected for this study to examine cell abnormalities, chromosome rearrangement, buds, nuclear bridges, MNi, necrosis, apoptosis, and chromosome loss (Fenech, 2006, 2007). Bleomycin was used as a DNA damage inducer in the micronucleus assay, and a mean of values from three healthy individuals treated with different concentrations of bleomycin showed that 0.75µg/ml caused the highest MNi frequency. The experiments in this assay were repeated from three different individuals healthy or patients, the results exhibited a normal nuclear division index (NDI), rated between 1.3 to 1.5 (Tables 10-11) with a normal value of (NDI) between one and two (Fenech, 2007). The percentage of bi-nucleated cells was estimated upon the examination of 1000 cells each concentration. The decline of multi-nucleated cells showed that the activity of Cytochalasin B was efficient and blocked cell division following the first division (Fenech, 2007). The effect of quercetin bulk and nano in the lymphocytes with DNA damage collected from healthy individuals was not significant with 10µM of the bulk form, whereas with the other concentrations it was significant with a value of $P \leq 0.001$ (Figure 45, Table 10). A comparison between the bulk and nano forms of quercetin treated lymphocytes from patients with TB (figure 46), showed significant DNA damage reduction with values *$P \leq 0.0281$ to**$P \leq 0.027$ and ***$P \leq 0.001$ with 10µM,

25μM, and 100μM, with the nano form being more efficient than the bulk form in reducing the occurrence of micronuclei. (Figures 46). The concentration of 100μM of the nano form of quercetin displayed a greater reduction in MNi development compared to the other concentrations (Figure 48), the reason for that quercetin can control oxidative stress resulting from phagocytosis process which resistance *Mycobacteria tuberculosis*. Furthermore, H_2O_2 is continuously produced by the macrophages that resulting high free radical loaded in pulmonary cells which may lead to cancer, quercetin has ability to scavenging free radicals that casing DNA damage, therefore small dose of quercetin nano could help to improve TB patient's immunity (Adebimpeet al., 2016).

8.4 Comparison between Results Obtained from the Comet and Micronucleus Assays

Overall, the quercetin-treated lymphocytes collected from the healthy individuals or the lymphocytes collected from the patients with TB both efficiently reduced DNA damage; however, they displayed different grades of this reduction, which depended on the quercetin form. The Comet assay results from experiments on lymphocytes showed that the nano form of quercetin reduced DNA damage more than the bulk form; however, the effect of the nano form of quercetin was observed to be significant (Figures 24 and 25) at any concentration $P \geq 0.05$. On the other hand, there was significant reduction in DNA damage in sperm collected from healthy individuals that were treated with the nano form of quercetin with maximum reduction observed at 100μM *$P \leq 0.001$ both for % Tail in DNA and Olive tail moment. This could indicate that the sperm is more sensitive in DNA damage than lymphocytes (Figures 32 and 33). In the micronucleus assay, both the nano and bulk forms of quercetin reduced the

number of MNi resulting from the bleomycin treatment in lymphocytes from healthy individuals. This reduction was more effective *** $P \leq 0.001$ at two concentrations (100 µM and 25 µM); and insignificant at 10 µM concentration for the bulk form (Figure 45). The possible reason for this could be the longer incubation time for the micronucleus assay compared to the Comet assay. This longer incubation time possibly allows the NPs to repair DNA damage via the penetration of the nuclear membrane. Moreover, the NPs have the ability to protect DNA because they can easily contact the nuclear components (Rasaie et al., 2014). A bulk form of quercetin can reduce genotoxicity at a certain level only due to its size. Specifically, the results from this study showed that bulk size can reduce DNA damage partially caused by H_2O_2 or bleomycin (Figure 45,46 and tables 10 and 11). In 2004, Oliveira et al. identified a weak interaction between quercetin and DNA in a bulk solution and these results are compatible with the perspective reported by Razavi (Prasain et al., 2016, Razavi et al., 2009).

Oxidative stress plays a major part in in the development of many chronic and degenerative illnesses (Reuter et al., 2010). Hydrogen peroxide is responsible for oxidative stress in TB patients due to phagocytosis and immune response. Several studies have examined how quercetin acts as a protective compound against Tuberculosis. The mechanisms of quercetin can inhibit DNA damage in TB patients by restricting the production of free radicals at high levels that induce DNA damage in a pro-oxidative state by causing chromosomal instability (Gibellini et al., 2010).

The mechanism of oxidative stress that causes genomic instability, gene expression dysfunction, and a rise in abnormal cell proliferation that can lead to cancer was described by Hanahan and Weinberg in 2011. Furthermore, DNA damage occur in cellular macromolecules including the lipid structures, due to increased oxidative stresses (Nordberg & Arner, 2001; Valko et al., 2007). Reactive oxygen species (ROS) can produce double-single DNA strand breaks and change the chemical structure of DNA by replacing purine and pyrimidine bases with 2′-deoxyribose (Hazra et al., 2007). It has been shown that DNA damage caused by oxidative stress could be a crucial cause of cancer and linked with the normal process of ageing (Bjelland & Seeberg, 2003). Furthermore, in order to determine quercetin genotoxicity, the effect of quercetin-induced lung cancer was investigated by the cell line NCL-H209 using flow cytometry and Western blot analysis to examine pro- and anti-apoptotic proteins, caspase -3 activities, and gene expression. The results showed that the proliferation of lung cancer was decreased in cells through G2/M, the capture of the cell cycle, and the encouraged apoptosis via a pathway called caspase-3 (Yang et al., 2005). Though, bleomycin can be used as an anti-cancer treatment it also increases the levels of oxidative stress that cause DNA damage (Dedon and Goldberg, 1992). The side effects of bleomycin could cause other complications, such as the recurrence of cancer, as a result of increased DNA damage (Cho et al., 2016). Therefore, it is important to control the oxidative stress when the cancer is being treated with bleomycin. As the natural compound quercetin has the ability to reduce oxidative stress, bleomycin can be used in combination with quercetin nano, which would be overall help reduce drug toxicity (El-Denshary et al., 2015).

8.5 The Effect of the Bulk and Nano Forms of Quercetin on Lymphocytes and Sperm-induced DNA damage by Heterocyclic Amines PhIP and IQ

The two food mutagens PhIP and IQ are heterocyclic amines created via the heating of proteinaceous food, and both compounds have been classified as genotoxic and carcinogenic (Durling and Abramsson-Zetterberg., 2005). In this section, the damaging DNA effects of various dietary and environmental compounds were studied by treating in vitro lymphocytes in TB patients in addition to sperm and lymphocytes in healthy donors. IQ and PhIP were used as DNA damage inducers in the lymphocytes collected from TB patients, and then the corresponding results were compared with the lymphocytes and sperm from healthy donors. Different concentrations of quercetin were mixed together with IQ or PhIP, and the DNA damage and protective potential of the flavonoid's quercetin were evaluated using the Comet assay. The results showed that 160μM of IQ and 140μM of PhIP were considered genotoxic doses (Figures 49, 50, 59 and 60) in the above of toxicity (Henderson et al., 2000). In the lymphocytes from the healthy individuals, the DNA damage resulting from the food mutagen effect treated with quercetin was significantly reduced to **$p \leq 0.0043$ and ***$P \leq 0.001$ when compared to the IQ- or PhIP-induced lymphocytes without quercetin treatment (Figures 51, 52, 61 and 62). The lymphocytes from the TB patients displayed greater DNA damage than those from the healthy individuals, and these higher levels of damage were also observed in the untreated cells from the in vitro treatment. Furthermore, it has been shown that quercetin has the potential to reduce DNA damage caused by the food mutagens IQ and PhIP. This reduction was observed to be significant in both

the Comet assay parameters of the % Tail DNA damage and Olive tail moment. The significance level ranged between *$p \leq 0.0405$ and ***P ≤ 0.001 based on the One-Way ANOVA test (Figures53, 54, 63 and 64). Kurzawa-Zegota et al, (2012) highlighted the fact that quercetin decreases oxidative stress in vitro caused by IQ and PhIP using lymphocytes from colon cancer patients and comparing the results with those of healthy individuals. The protective effect of quercetin on PhIP- and IQ- induced DNA damage in the spermatozoa of healthy individuals, were evaluated using Comet assay showed significant DNA protection. The DNA damage was reduced when 10 μM, 25μM, and 100μM of quercetin (in both forms) was introduced; furthermore, the significance value was between * P ≤ 0.00405, **$p \leq 0.0031$, and ***P ≤ 0.001, respectively. Anderson et al. (1998) noted that the flavonoids were examined in combination with IQ via the Comet assay for lymphocytes and sperm in healthy individuals. These results suggested that the flavonoid compounds produced antigenotoxic effects, since DNA damage was reduced in both the sperm and lymphocytes. Since, dietary supplements containing flavonoids from fruits and vegetables could help in protecting DNA against oxidative stress, it is simple to implement anti-mutagens into the daily diet in order to decrease the progression of cancer, stimulate cells into apoptosis, and remove mutant cells (Ferguson et al., 2004). Spermatozoa exhibited greater baseline DNA damage than the lymphocytes from healthy individuals. In addition, the DNA damage and abnormal chromosomal structure were much greater in the haploid cells than in the diploid cells (Genesc et al., 1990). In conclusion, quercetin was shown to decrease oxidative stress resulting from IQ and PhIP in vitro treatment. The data indicated that 25 μM or 100μM of quercetin nano could be a useful protective

compound to decrease DNA damage resulting from oxidative stress in patients with TB.

8.6 The Effect of Both Quercetin Forms on COX1, COX2, P53, and Bcl-2 Gene Expression in Lymphocytes from Healthy Individuals and TB Patients Using Real Time PCR

COX1 and COX2 are enzymes that play an important role in the production of prostaglandin-endoperoxide. The prostanoids are vital biological mediators that assist in different biological processes. The main role of COX1 and COX2 enzymes is to transform arachidonic acid into prostaglandin H2, a process that ends with synthesising the biological form of prostaglandin, which has a function in cell division and apoptosis (Ulrich et al., 2006). Marakalala et al. (2016) analysed the proteomes of granulomas in TB patients using laser-capture microdissection, mass spectrometry, and confocal microscopy and then created detailed molecular maps of human granulomas. In cavity and caseous granulomas, the COX1 and COX2 were diffusely expressed in the cellular layers, although they were primarily absent in the caseous granulomas; however, COX1 and COX2 expression was detected in fibroblasts and macrophages (Marakalala et al., 2016). In this section, this study examined the influence of quercetin nano and bulk forms on the expression of COX1 and COX2 in lymphocytes of TB patients and compared the results with those of healthy individuals. The data exhibited a non-significant change in the expression of COX2 genes in the lymphocytes from both healthy and TB-affected individuals following two hours of treatment with the bulk and nano forms of quercetin. (Figures 71-72). Ramyaa et al. (2014) studied the protective effect of quercetin by examining HepG2 cells induced oxidative stress, the

results of which illustrated the down-regulation of NF-κB and COX-2 and the up-regulation of Nrf2 expression. However, COX1 gene expression was down-regulated by both forms of quercetin (Figures 69-70). In the lymphocytes from healthy individuals, the bulk form of quercetin downregulated the COX1, which was not significant compared to the untreated cells. On the other hand, the lymphocytes from the TB patients showed significant down-regulation of COX1 with both the bulk and nano forms of quercetin with significance values between $*p \leq 0.045$ and $**p \leq 0.0074$, respectively. When the results of nano form was compared to the untreated cells and 140μM concentration of PhIP, the significant down-regulation of COX1 was observed in the lymphocytes from healthy individuals and patients with TB. This perspective reflects the idea that the large surface area of the nano form could explain the difference in DNA repair between the two quercetin forms, expanding upon the notion that perhaps the nano size allows for the more rapid penetration of the nucleus compared to the bulk size (Huang et al., 2012).

Regarding the p53 gene expression, there was no significant up-regulation of gene expression via the quercetin treatment of bulk and nano forms with lymphocytes from patients with TB and healthy individuals. Because quercetin activated p21 which supports cell cycle arrest. Tanigawa et al. (2008) illustrated that the stabilization of p53 is involved in the quercetin-induced cell cycle arrest and apoptosis using the Hep G2 cell line (i.e., hepatocyte carcinoma). They attempted to reactivate p53 in HepG2 cell line via the implementation of quercetin. The data demonstrated that quercetin prevents the proliferation of HepG2 cells through the incitement of cell cycle arrest and programmed cell death. Additionally, they found that at the molecular level, the quercetin

encouraged p53 phosphorylation and total p53 protein; however, it did not up-regulate p53 mRNA at the transcription level. Subsequently, quercetin up-regulated the p21 gene expression and blocked the cyclin D1 expression in support of cell cycle arrest (Tanigawa et al., 2008). By comparing the estimation of dose-dependent differences, the effects of quercetin and curcumin were investigated in relation to treating double-strand break DNA damage from two natural polyphenols: quercetin (QUE) and curcumin (CUR), 3 μM curcumin, and 8 μM quercetin, which showed a statistically significant increase in p53 activation that was similar in magnitude to both chemicals at lower doses. However, at the highest dosage, more than10 μM curcumin and 30 μM quercetin, the cells were directed to apoptosis (Sun et al., 2013).

The BCL-2 gene is in the B cell lymphoma family and plays an essential role in regulating apoptosis by controlling intracellular mechanisms such as pro-apoptotic and anti-apoptotic signals. In cancer, apoptosis avoidance caused by the malfunctioning of the Bcl-2 gene is a recurring phenomenon, and, as a result, a decrease in specific anti-apoptotic Bcl-2 protein could indicate an essential treatment opportunity (Ashkenazi et al., 2017). In this respect, this study examined the effect of quercetin bulk and nano forms on Bcl-2 gene expression. This effect was investigated during a two-hour incubation period using 140 PhIP as a DNA damage inducement in the lymphocytes of both healthy individuals and patients with TB. The results showed that significant down-regulation of Bcl-2 gene expression was evident in the nano form of quercetin, with a P value of *$p \leq 0.0401$ in the lymphocytes from healthy individuals and **$p \leq 0.0060$ in the lymphocytes from patients with TB, whereas the bulk form displayed a non-significant effect. These findings were compatible

with a study on HepG2 cell line in hepatocellular carcinoma patients and treated with quercetin that investigated the influence of quercetin on the activation of the apoptotic pathway in the HepG2 cell line. The cells were treated for 18 hours, and the vast majority of these cells were dead in a dose-dependent manner. At the time of incubation, the quercetin-induced apoptosis occurred via the activation of caspase -3 and -9. Furthermore, the quercetin reduced the Bcl-xL and Bcl-xs ratio and amplified the translocation of Bax to the mitochondrial membrane (Granado-Serrano et al., 2006). Gibellini et al. (2010) highlighted that quercetin activates programmed cell death through the mitochondria pathway, beginning with the loss of the mitochondria membrane and the release of the cytochrome c from the mitochondria to the cytosol and ending with the stimulation of the caspase-3 and caspase-7. Moreover, the quercetin encouraged the up-regulation of Bax and Bak that cause the downregulation of Bcl-2 and Bcl-x (Gibellini et al., 2010). The results of this project were in accordance with the perspective that quercetin plays a promising role in reducing oxidative stress which causing cancer. Moreover, as the survival of virulent *mycobacterium tuberculosis* depends on prevention of apoptosis of pulmonary cells through upregulation of Bcl-2, quercetin with its ability to down regulate Bcl-2 can play an important role in slowing the progression of TB (Zhang et al., 2013).

8.7 Investigation of the Catalase Protein in Lymphocytes from Healthy Individuals and TB Patients Treated with Both Forms of Quercetin Using Western Blot Analysis

Catalase is a common enzyme able to breakdown hydrogen peroxide (H_2O_2) into water and oxygen. This antioxidant enzyme is produced in all main body

organs, particularly in blood cells, the liver, and the kidney. In these organs, catalase plays a crucial role in cell defence against oxidative stress (Glorieux et al., 2018). This study examined the effect of the two forms of quercetin on the expression of catalase protein in lymphocytes from both healthy individuals and TB patients using the Western blot method. Two-hour incubation times were selected for each treatment. In regard to the catalase protein expression in the lymphocytes of healthy individuals the 140μM of PhIP was used as a DNA damage inducer (Figures 59 and 60), the results illustrated that both forms of quercetin when compared to the untreated cells and140μM of PhIP was not significant in regard to the lymphocytes collected from the healthy individuals (Figure 77). Conversely, the nano form of quercetin displayed a significant change in catalase protein expression during the same period of incubation *$p \leq 0.0236$. Furthermore, the increase in the protein expression following quercetin treatment using lymphocytes from healthy individuals could explain the minor increase in DNA damage that was observed by the Comet assay in chapter 3 (Figures 24 and 25). In the lymphocytes from patients with TB, the catalase protein expression was not found to be significant for the bulk form of quercetin, whereas significant protein expression was observed in the nano form of quercetin. These results were in accordance with the study published by Singh (2009), highlighting that NPs possess stable and recognized chemical and physical properties in their normal size. However, it should be noted that the smaller size of NPs permits them to penetrate the cell membrane in several ways and helps them diffuse through the nuclear membrane. During the mitosis stage, NPs can become enclosed by the nucleus, since the nuclear membrane disintegrates during this stage. This, in turn, can lead to a direct reaction

occurring between the NPs and chromosomes that can then promote contact with DNA (Singh et al., 2009). Furthermore, this knowledge may explain the DNA damage reduction that was detected by the Comet assay experiments in chapter 3 (Figures 36 and 37), also demonstrated the reduction of DNA damage resulting from oxidative stress during phagocytosis against *Mycobacterium tuberculosis* and support the role of quercetin as a nutritional supplement in TB patients.

8.8 Conclusion

Data from the Comet assay indicated that the nano form of quercetin produced a significant reduction in DNA damage resulting from oxidative stress that caused by H_2O_2 in the lymphocytes of patients with TB and sperm from healthy individuals. However, this significant reduction in DNA damage was showed with the nano form compared to the positive control. Moreover, the micronucleus assay results displayed a larger decrease in MNi frequency and DNA damage compared to those of the Comet assay. This is probably due to increased incubation times, since the micronucleus assay tracked a comparable pattern to that of the Comet assay data. In both techniques, the nano form of quercetin was more effective than the bulk form in reducing DNA damage and MNi frequency. In regard to the examination of gene expression, both types of quercetin were observed significant down-regulate to the expression of COX1. The Bcl-2 gene was significantly down-regulated with the nano form. This could be the possible role of quercetin to guide the lymphocytes to apoptosis. Western blot analysis also found that the catalase protein expression was significantly up-regulated by the nano form of quercetin in the lymphocytes of patients with TB, whereas the up-regulation was not significant with the bulk

form. The data from this study as compatible with the perspective that flavonoids such as quercetin can be linked to having health-promoting effects due to their anti-oxidative, anti-inflammatory, anti-mutagenic, anti-carcinogenic, and enzyme modulating properties.

In conclusion, quercetin is responsible for the downregulation of the Bcl-2 expression that could adversely affect the survival of the mycobacterium and it simultaneously upregulates the catalase protein to reduce the oxidative stress which is prevalent during chronic illnesses. This combined with the genoprotective effect seen in the Comet assay and micronucleus assay suggests a possible role for quercetin, especially the nano form, in genoprotection in lymphocytes from patient with TB through its enzyme modulating properties and genoprotective effects.

8.9 Future Work

Based on this discussion and the above-mentioned findings, it would be valuable that examining the effect of quercetin studied extensively and using further methods to confirm these data. Micronucleus FISH (Fluorescence in situ hybridisation) is one of these methods which permit the assessment of the nature of chromosome damage lesions. It would be interesting if this work could be repeated with lymphocytes from different types of respiratory disease to determine whether the results will be similar or different compared to patient with TB. Other cancer types, such as Leukaemia, and breast cancer, are suggested to establish whether similar responses could be obtained. Similar or differing genetic end-points using qPCR could be investigated, including Bcl-xl,

BAX, BAK, P21, and P38. The gene expression and DNA repair studies are known to be associated with oxidative stress and cell cycle arrest. Caspase-3 and caspase-7 can be linked to the cytochrome c level to investigate apoptosis through the mitochondria pathway. Superoxide dismutase enzymes SOD1 and SOD2, which are responsible for destroying free superoxide radicals in the body, can also be investigated using western blot techniques to detect protein expression.

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10 Appendix

Appenix I Blood consent form

UNIVERSITY OF BRADFORD
MAKING KNOWLEDGE WORK

Consent form for patients with TB and healthy individuals

Title of Project: **The genotoxic effect in respiratory disease of Tuberculosis (TB) patients compared to healthy controls in diploid lymphocyte and haploid sperm cells, after treated with heterocyclic amines and quercetin bulk and nano form**.

Reviewed by Leeds Central Research Ethics Committee (REC) (REC reference number: 12/YH/0464)

1. I confirm that I have read and understand the information sheet (version 3, 19- 06-09) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights beingaffected. ☐
3. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from the NHS Trust or the University of Bradford, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐
4. I agree that the sample I have given, and the information gathered about me can be stored at the University of Bradford, as described in the attached information sheet. ☐
5. I agree to take part in the above study. ☐

Name of Patient/donner	Date	Signature
Name of Person taking	Date	Signature

Appendix II Invitation to the research study

UNIVERSITY OF BRADFORD
MAKING KNOWLEDGE WORK

Participant Information Sheet for patient and healthy volunteer
Reviewed by Leeds Central Research Ethics Committee (REC)
(REC reference number: 12/YH/0464)

Invitation to the research study

We would like to invite you to take part in a research study. Before you decide you need to understand why the research is being done and what it would involve for you. Please take time to read the following information carefully. Talk to others about the study if you wish to and you will be allowed around 24hours to consider this.

(**Part 1** tells you the purpose of this study and what will happen to you if you take part.

Part 2 gives you more detailed information about the conduct of the study).

Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether you wish to take part.

Part 1

What is the purpose of the study?
In this study white blood cells will be treated in a test tube with very small chemical particles to determine if patients with cancerous and inflammatory diseases are more at risk after exposure. A blood sample of around 6-8 teaspoons (40 ml) will be taken. Samples will be stored only for the duration of the study and used for studies of a similar nature or to check original responses. The research is for a PhD programmed involving post-doctoral fellows and PhDs.

Why have I been invited?
You have invited because you have disease states and we should like to determine if these small chemical particles could be more harmful to you than to people without diseases state than those without such disease.

Do I have to take part?
It is up to you to decide. We shall outline the study and go through this information sheet, which we shall then give to you. We shall ask you to sign a consent form to show you have agreed to take part. You are free to withdraw at any time, without giving a reason. This would not affect the standard of care you receive.

Part 2
What will happen to me if I take part?
A single blood sample will be taken, and you will not need to attend the Clinic again for this research study. A brief questionnaire will need to be completed by the researchers.

We shall need to access your notes so that they can be linked in an anonymous way to your clinical data which can be tied up with the research results. Everyone will be given a coded study number.

The data obtained will only be available to the research team and will **not** be returned to you. Responses will be compared only on group basis i.e. collective responses from patients with chest diseases compared to collective responses from people without chest diseases. Results could be published in the form of scientific papers. The work will benefit the medical and scientific community at large but will not be of direct benefit to you as an individual. If, however, you would like more information, Dr BK Jacobs will be prepared to talk to you individually about study results.

People who cannot take part in the study.
People who are not well enough to take part will be excluded (e.g. those with anaemia) If you have any further questions, you could contact the research team:
Professor Diana Anderson, Established Chair in Biomedical Sciences, BSc MSc PhD DipEd FSB, FATS, FRC Path, FIFST, FBTS, FRSM, FHEA, FRSC, University of Bradford Richmond Road, Bradford, West Yorkshire BD7 1DP, United Kingdom and Honorary Research Consultant to Bradford NHS Trust. Email: d.anderson1@bradford.ac.uk.

Dr Mojgan Najafzadeh. Division of Medical Sciences, University of Bradford, Richmond Road, Bradford, BD7 1DP and Honorary Research Consult

Appendix III volunteer's information

UNIVERSITY OF BRADFORD
MAKING KNOWLEDGE WORK

1.1.1 DATA COLLECTION FORM

(To be completed by the Doctor)

REVIEWED BY LEEDS East RESEARCH ETHICS COMMITTEE (REC)
(REC REFERENCE NUMBER: (12/YH/0464)

PATIENT NUMBER ☐ DATE OF SAMPLE ☐

AGE ☐

SEX (PLEASE TICK) M F CONSENT INFORMATION SHEET Y / N
ETHNIC GROUP Y / N

OCCUPATION ☐

CURRENT SMOKER Y / N PAST SMOKER Y/N ☐
CIGARETTES HOW MANY/MUCH PER Y /NDAY?
ALCOHOL Y / N CIGARS

DIET Western Asian OMNIVORE | VEGETARIAN VEGAN |

VITAMINS / ANTI-OXIDANTS (PLEASE LIST) ☐

PRESCRIBED DRUG USE (PLEASE LIST) ☐

RECREATIONAL DRUG USE Y/N

IF YES PLEASE LIST

MEDICAL

Cancer inflammatory disease

Extent site

CANCER	
Inflammation diseases	
Precancerous state	
Other medical conditions please list	
Family history of cancer and inflammatory disease	
Chemotherapy or radiotherapy	

Appendix IV Table 1 Brief information about the blood samples collected from healthy donors.

No	Sample code	Age	Ethnicity	Gender	Smoking history	Family history
1	KE27.8.15	47	Asian	Male	Smoker	-
2	MS 27.8.15	42	Arab	Male	Smoker	-
3	HS 27.8.15	55	Brattain	Male	Non-smoker	-
4	MA17.6.2015	45	EU	Male	Smoker	-
5	DN25.8.15	47	Arab	Male	Non-smoker	-
6	MS23615	35	Asian	Male	Non-smoker	-
7	M 23.3.15	56	Arab	Male	Non-smoker	-
8	A 10.3.15	37	Asian	Male	Non-smoker	-
9	CK12.5.15	26	Asian	Male	Non-smoker	-
10	1PA0 27.01.2016	41	Asian	Male	Non-smoker	-
11	1PA18.3.2016	40	Arab	Male	Smoker	-
12	1PA2 26.4.2016	34	EU	Male	Smoker	-
13	1PA3 11.72016	42	Asian	Male	Smoker	-
14	1PA4 17.8.2016	34	Arab	Male	Non-smoker	-
15	1PA5 06.9.2016	40	Asian	Male	Non-smoker	-
16	1PA6 02.9.2016	38	Asian	Male	Smoker	-
17	1PA7 15.9.2016	32	English	Male	Smoker	-
18	1PA8 21.9.2016	36	Arab	Male	Smoker	-
19	1PA9 21.9.2016	33	Arab	Male	Non-smoker	-
20	1PA10 21.9.2016	48	Arab	Male	Non-smoker	-

Appendix V Table 2 Brief information about the blood samples collected from patient with TB used in the Micronucleus assay.

No	**Sample code**	**Age**	**Ethnicity**	**Gender**	**Smoking history**	**Family history**
1	RAE0816990	56	Bratish	Male	Non-smoker	--
2	9576	49	Bratish	Male	Non-smoker	--
3	9577	53	Bratish	Male	Non-smoker	--

Appendix VI Table 3 Brief information about the blood samples collected from patient with TB used in the Comet assay.

No	Sample code	Age	Ethnicity	Gender	Smoking history	Family history
1	TB1.27.4.15	55	Asian	Male	Non-smoking	---
2	TB2.27.4.15	48	British	Male	smoking	---
3	TB 1.6.15	39	Asian	Male	Non-smoking	---
4	TB 27.7.15	41	Asian	Male	Non-smoking	---
5	TB 3.8.2015	57	Asian	Male	smoking	---
6	TB2 27.7.15	58	Arab	Male	Non-smoking	---
7	TB2 27.4.15	49	EU	Male	Non-smoking	---
8	TB 3.5.15	52	Asian	Male	Non-smoking	---
9	TB 6.10.15	55	Asian	Male	smoking	---
10	TB1 26.1.15	58	Asian	Male	Non-smoking	---

Appendix VII Table 4 Brief information about the blood samples collected from patient with TB used in the food mutagens PhIP and IQ.

No	Sample code	Age	Ethnicity	Gender	Smoking history	Family history
1	TB2.23.3.15	55	Asian	Male	Non-smoking	---
2	TB3.9.3.15	48	British	Male	Smoking	---
3	TB1 3.2.15	39	Asian	Male	Non-smoking	---
4	TB2 3.02.15	41	Asian	Male	Non-smoking	---
5	TB1 3.2.15	57	Asian	Male	Smoking	---
6	TB 27.7.15	58	Arab	Male	Non-smoking	---
7	TB 27.4.15	49	EU	Male	Non-smoking	---
8	TB2 24.1.15	52	Asian	Male	Non-smoking	---
9	TB3 6.10.15	55	Asian	Male	Smoking	---
10	TB2 26.1.15	58	Asian	Male	Non-smoking	---

Appendix VIII Table 5 Brief information about the blood samples collected from patient with TB used in the QPCR and Western blot.

No	Sample code	Age	Ethnicity	Gender	Smoking history	Family history
1	MRN0161282	41	Asian	Male	Non-smokikg	---
2	1453780	38	British	Female	Non-smokikg	---
3	1385793	28	Asian	Female	Non-smokikg	---
4	1242606	36	British	Female	Non-smokikg	---
5	1152271	32	Asian	Female	Non-smokikg	---

Appendix IX Consent form was used to collect sperm form healthy individuals donated sperm samples.

Sperm consent form

University of Bradford

Department of Biomedical Sciences

Richmond Road

Bradford, BD7 IDP

United Kingdom

Project Title: The genotoxic effect in respiratory disease of Tuberculosis (TB) patients compared to healthy controls in diploid lymphocyte and haploid sperm cells, after treated with heterocyclic amines and quercetin bulk and nano form.

Volunteer's Donor Code: _____

Date: _____

1. I hereby authorise Prof. D. Anderson or her assistants, Dr. A. Baumgartner and Dr. E. Cemeli, to receive semen samples from me to be utilised for genotoxic in vitro experimental purposes (but NOT for in vitro fertilisation-type procedures or reproductive cloning).
2. I understand that the procedure for obtaining samples is standard and not harmful.
3. I understand that there is no obvious risk associated with the procedure.
4. This activity does not involve medical treatment.

5. I understand that I will not be affected in any way by my decision to participate or not.
6. I understand that the purpose of performing these procedures is to obtain semen for biomedical and biochemical tests outside the human body. Specifically, the material will be used for the following procedures:
   - to develop various new methods for detecting defective sperm, - to evaluate food mutagens and in future other chemicals acting 'in vitro' on the sperm DNA/chromatin - to become part of an archive of frozen and coded semen samples.

   I understand that semen is stored and frozen. However, the sperm will never be used for fertilisation or reproductive cloning experiments.

7. I understand that this study will result in no direct benefit to me but it may contribute to the understanding of the possible effects of various chemicals for producing DNA damage in sperm. These findings may therefore be of some benefit to all of us and our children in the future.

8. I understand that Prof. D. Anderson or her assistants will encode the sample to protect my identity and will not disclose my name to other individuals. I will be assigned a random donor code that will be used for all of my samples. Only the above person(s) will know that I am a participant in this study and what my code is. Personal data will be kept in accordance with data protection laws.

9. I understand that Prof. D. Anderson or her assistants will answer any inquiries I may have at any time concerning the procedures and/or investigation. Her address and phone number are indicated in item 14 below.

10. I understand that Prof. D. Anderson or her assistants cannot disclose any possible findings to me concerning my own sample.

10. Any publication arising from this study will be made without specific reference to my name.

1 1. I recognise that my participation in this experiment is entirely voluntary and I may refuse to participate or may withdraw at any time without jeopardy. Owing to the scientific nature of the study, the investigator may at his/her absolute discretion terminate the procedures and/or investigations at any time.

12. I acknowledge that, if I wish, I can keep the copy of the consent form, which is provided.

13. Prof. D. Anderson is responsible for the conduct of the research in which I am to participate. This research is sponsored by the European Union (NewGeneris contract no. 016320-2). Laboratory analyses will be done in the University of Bradford, Division of Biomedical Sciences, Bradford, UK.

14. I understand that if I have any complaints or concerns about the procedures, I may address them to Prof. D. Anderson or her assistant(s), in person, by telephone, or in writing. Prof. D. Anderson can be reached by phone at 01274-23-3569, by Fax at 01274-30-9742, by email at d.andersonl@bradford.ac.uk or by mail at the University of Bradford, Devision of Biomedical Sciences, Richmond Road, Bradford, BD7 IDP, UK.

Volunteer's signature: _____ Date _____

Appendix X Reproductive health questionnaire was used to collect sperm form healthy individuals.

Reproductive Health Questionnaire

Donor ID

_____

START HERE Tick the boxes ZI of your response or fill in the blank

---

1. What is your age? _____ years

2. What is your weight? kg / lbs

3. What is your height? cm / in _____

---

4. Which of the following best describes your ethnic background? Asian

☐ Indian, C] Pakistani, Chinese

Black

☐ Caribbean, CI African, Afroamerican

Caucasian

☐ West-european, East-european,

☐ Mediterranean, CI American

O Other or mixed ethnic background

Please specify:

_____

5. Do you have children? ☐ Yes

If yes: ☐ No

How many? _____

How old are they?

You can provide further details about your ethnic background if you wish

Are they healthy? ☐ Yes ☐ No

6. Have you ever had difficulties fathering a child? ☐ Yes ☐ No

If yes, explain (when):

7. How would you describe your sexual activity in the last three months? ☐ more than 5 times per week ☐ 3-5 times per week ☐ 1-3 times per week Cl less than once per week

1

8. What is your current or most recent job title?

9. How long have you been working in this job?

1 0. Do you currently smoke, are you an ex-smoker, or have you ever smoked cigarettes, cigars or pipes regularly?

If current smoker:

How many cigarettes did you smoke?

How many years have you been smoking?

If ex-smoker:

When was the last time you smoked?

How many cigarettes did you smoke?

1 1. On average, how many glasses of wine, pints of beer or mixed

drinks with hard alcohol do you drink each week?

12. On average, how many cups of caffeinated coffee, tea or beverage do you drink each day?

13. Do you take multivitamins or vitamin supplements four or more times a week?

If yes:

Which one?

_____

_____ _____

years months

☐ Yes, current smoker ☐ No, ex-smoker

☐ No, never smoked

_____cigarettes per day (in last 3 months) years

_____

_____ months or years ago

_____

cigarettes per day

_____

drinks per week (in last 3 months)

_____

_____cups (cans, drinks) per day (in last 3 months)

C] Yes

☐ No

Cl Vitamin C

Cl Vitamin E

☐ Combination of Vit.C and E

C] Others:

_____

14. During the last year have you had any notable exposure to.. Yes No Maybe

a. radiation? ☐ ☐ ☐

b. pesticides? ☐ ☐ ☐

c. solvents? ☐ ☐ ☐

d. chemotherapy? ☐ ☐ ☐

e. lead? ☐ ☐ ☐

f. drugs like marijuana, heroin,.? ☐ ☐ ☐

g. other hazardous materials? ☐ ☐ ☐

specify:

If yes: Please give details:

15. Do you have any chronic health problem(s)? ☐ Yes ☐ No

If yes:

What are they (please list)

16. Have you had any other illness in the past three months? ☐ Yes ☐ No

If yes:

What were they (please list)

What medication or treatments do you take for this health problem(s)?

What medication or treatments did you take for the illness (drug + dose)?

17. Have you had any fever in the past 3 months? ☐ Yes ☐ No

If yes:

When was the most recent fever?

How high was your temperature? day OC month year

18. Have you ever had any of the following procedures or conditions? Yes No

a. Vasectomy

If yes, when? _____ / _____

month year

b. Undescended testicle If yes, when treated? _____ / _____

month year

c. Varicocele (varicose veins of the scrotum)

If yes, when treated? _____ / _____

month year

d. Other urinary or genital conditions Specify:

_____

When treated? _____ / _____

month year

e. Cancer

Specify type of cancer:

_____

Year of diagnosis:

_____

1

f. Chemotherapy for cancer

Start of therapy: _____ End of therapy:

_____

g. Radiation therapy for cancer

Start of therapy:

_____

End of therapy: _____

h. X-ray diagnosis during the last year What part of the body?

_____

How many films were taken?

_____

When? _____ / _____

month year

---

19. We would like to ask you about your level of stress — at present, how would you rate your satisfaction with....

very unsatisfied <<--OK-->> very satisfied

.	.. yourself?	10	20	30	40 50
.	.. your family?	10	20	30	40 50
.	.. your friends?	10	20	30	40 50
.	.. where you live?	10	20	30	40 50
.	.. your job?	10	20	30	40 50
	(other:)_____	10	20	30	40 50

---

20. Regarding your sample, how long was your sexual abstinence? days

---

i. Date (questionnaire completed) _____day month year

Thank you for your time answering the questions. We greatly appreciate your effort.

Appendix XI date sheet analysis was used to investigate sperm form healthy individuals.

Semen Analysis Protocol and Data Sheet		
Prerequisites before semen examination Collect samples after a minimum of 3 days of sexual abstinence. Label the container with date and time of the collection and the anonymous donor code. Avoid extreme temperatures (<20, >40^0 C) during any transport of the semen. The donor's questionnaire has to be finished. Let the semen liquify for 1 hour at room temperature. Mix the semen thoroughly in the container. Finish the semen examination within 2 hours after collection.(Please attach anv raw data sheets to this form.)		
Donor Code: _____	Date: _____ Abstinence: _____days	Time of Sampling: _____ Time of Analysis: _____
Tick the boxes with the answer or fill in the result:		
A) General Impression	normal (grey-opalizing, homogeneous) abnormal (brown when red blood cells are present,etc.)	
B) Volume	_____ml	
C) Consistence (viscosity)	normal abnormal Submerge a glass rod into the semen and pull it slowly out. If a thread could be drawn longer than 2 cm then note it as abnormal.	
C) pH	_____ Put a drop of semen onto a pH-paper and wait for 30 seconds. The normal pH-range should be between 7.2 and 8.0.	

D) Motility	% fast-progressive Number of cells evaluated: _____ % slow-progressive % non-progressive, but tail movement _____% immobile, no tail movement Characterise on 2 different slides 100 sperm cells (slide on a 37 °c slide warmer, 10 pl semen covered with a 22x22 mm cover-slip, let it sit for 1 minute after cover-slipping, standardised depth of 20 pm, magnification of 200-600x). Calculate the mean. If the results differ by more than 10 %, characterise a third slide.
E) Morphology	Number of cells evaluated: _____ _____% normal % abnormal Normal sperm: oval head with a acrosomal region. No head, midpiece, or tail defects. Cytoplasmic drops smaller than a 1/3 of a normal head. Characterise on 2 different slides 100 sperm cells each. Calculate the mean. If the results differ by more than 10 %, characterize a third slide.
F) Sperm Concentration	_____ million per ml Dilute 50 pi semen (1:20) in 950 pi NaHCOdformalin (5g/100ml NaHC03, Iml 35% v/v formalin). Mix well, put a drop onto each grid of a cell-counting chamber, cover-slip it. Let it sit for 5 min. Use phase-contrast. On both grids, count only normal sperm with tails in two of the four outer fields (each consisting of 4x4 squares). Calculate the sperm concentration. Neubauer-Improved chamber: = n • x DF = n • 5000mr^{l} • 20 = n • 100,000mr^{l} = sperm conc. [$10^{6}mr^{l}$], n = mean of the two scored grids, x = 5000ml-l - extrapolation factor to reach vol. of 1 ml, DF = 20 - dilution factor for a 1:20 dilution) [an 4x4 outer field has an area of lmm^{2} with a depth of 0.1mm, which equals a volume of 0.1 Pl]

Appendix XII Table Brief information about the sperm samples collected from healthy individuals

No	Sample code	Age	Ethnicity	Smoking history	Family history
1	AG 03042016	42	Arab	Non-smoking	No
2	M210052015	38	Asian	Non-smoking	No
3	HITH24102015	41	Arab	Non-smoking	No
4	MH217112015	35	Slovakian	Non-smoking	No
5	EN30112015	32	Turkish	Non-smoking	No
6	M428082015	47	Asian	Non-smoking	No
7	GA28112016	48	Polish	Non-smoking	No
8	M102212205	43	Arab	Non-smoking	No
9	EN30112015	33	Asian	Non-smoking	No
10	M325082015	42	Asian	Non-smoking	No
11	MM01122015	46	Polish	Non-smoking	No
12	AM1512206	28	Spanish	Non-smoking	No
13	ME08122015	35	Asian	Non-smoking	No
14	M1022122015	42	Arab	Non-smoking	No
15	EA16012016	46	Asian	Non-smoking	No
16	EABDUL18116	37	Arab	Non-smoking	No
17	AG23012016	29	Asian	Non-smoking	No
18	FB1222016	36	Asian	Non-smoking	No
19	MB1822016	41	British	Non-smoking	No
20	AR25012016	27	Polish	Non-smoking	No